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THE COMPONENTS OF INHERITANCE OF
ADRENAL CORTICOSTERONE SYNTHESIS IN MICE

by

PETER RORY WELLS WOOD

Ph.D. thesis submitted, in April 1982,
to the Science Faculty of Glasgow University,
based on research carried out at the
Genetics Institute, Church Street, Glasgow.

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DEDICATION

A paltry recompense for all the loving support, despite
her many hours of loneliness, I have received from Morag.

Before BIG BANG, a humble god
whose strength is singularity;
no clash of Marx with selfish gene
in His united firmament.

or phrased another way, by Schlegel:

Through every sound there sounds,
Within earth's coloured dream,
One quiet sound drawn out
For him who listens secretly.

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TABLE OF CONTENTS

	<u>Page No.</u>
DEDICATION	i
ACKNOWLEDGEMENTS	ii
TABLE OF CONTENTS	iv
LIST OF TABLES	xii
LIST OF FIGURES AND PLATES	xxii
LIST OF ABBREVIATIONS	xxiv
ABSTRACT	xxv
CHAPTER 1 INTRODUCTION	1
CHAPTER 2 MATERIALS AND METHODS	15
2.1 ANIMALS	15
2.1.1 Strains used	15
2.1.2 Abbreviations for genetic- ally defined stocks	17
2.1.3 Husbandry	18
2.1.3.1 Breeding of pure strains	18
2.1.3.2 Caging prior to experiment- ation	19
2.2 STEROID EXTRACTION AND ANALYSIS	20
2.2.1 Introduction	20
2.2.2 Glassware preparation	21
2.2.3 Corticosterone standard storage	22
2.2.4 Steroid extraction from steroidogenic incubates	22
2.2.5 Thin layer chromatography	24
2.2.6 Authentication of corticost- erone in unchromatographed methylene chloride extracts	26
2.2.7 Fluorometric assay of corti- costerone	27
2.2.7.1 Characterisation of assay	28
2.2.7.2 Assay procedure	30

Page No.

2.3	CELL COUNTS USING COULTER COUNTER	33
2.3.1	Introduction	33
2.3.2	Haemocytometer counts, and Coulter counts using an appropriate threshold setting	34
2.3.3	Evidence suggesting different counting characteristics of a replacement Coulter counter	40
2.3.4	Threshold setting alteration or use of a correction factor to render counts from the replacement Coulter counter comparable with those of the original counter	46
2.3.4.1	Selection of a new threshold setting on the replacement counter	46
2.3.4.2	The correction of counts made at threshold $T = 21$ on the replacement counter	49
2.4	ELECTROPHORETIC IDENTIFICATION OF ISOCITRATE DEHYDROGENASE (<u>Idh-1</u>) AND MALIC ENZYME (<u>Mod-1</u>) ISOZYMES	51
2.4.1	Introduction	51
2.4.2	Buffer solutions	51
2.4.2.1	Gel and tank buffer	51
2.4.2.2	Staining buffer	52
2.4.3	Preparation of liver homogenates	52
2.4.4	Preparation of starch gels and running of samples	52
2.4.5	Staining of gels	54
2.4.5.1	Stains	54
2.4.5.2	Isozyme recognition	55
2.5	IMMUNO-ELECTROPHORESIS	56
2.6	DRIED ORGAN WEIGHTS	57
2.7	COAT COLOUR MARKER GENES	58
2.7.1	Backcrosses of C57 x DBA F_1 's to DBA	58
2.7.2	Backcross of CBA x DBA F_1 to DBA	58

Page No.

CHAPTER 3	THE ADRENAL DISSOCIATION AND INCUBATION TECHNIQUES	60
3.1	INTRODUCTION AND HISTORY OF ADRENAL DISPERSION	60
3.1.1	Introduction	60
3.1.2	The history of the isolated adrenal cell technique	62
3.2	MOUSE ADRENAL DISPERSION AND ACTH INCUBATION	64
3.2.1	Composition and preparation of buffers and stock solutions	64
3.2.2	Pooled adrenal dispersion and ACTH incubation	66
3.2.3	Modifications used to disperse individual adrenal pairs	70
3.2.3.1	Enzymic modifications	72
3.2.3.2	Mechanical modifications	74
3.2.4	Mechanised dissociation	78
3.2.4.1	Apparatus for mechanised dissociation	78
3.2.4.2	The mechanically assisted dissociation procedure	81
3.2.5	Complete protocol for the dispersion and steroidogenic incubation of single adrenal pairs	83
3.3	THE INHIBITION OF STEROIDOGENESIS IN RESPONSE TO ACTH BY CONTAMINANTS OF BOVINE SERUM ALBUMIN	85
3.3.1	Introduction	85
3.3.2	Association of steroidogenic inhibition with the use of different batches of BSA	87
3.3.3	Reactivity of cells to dbcAMP in experiments with inhibited ACTH responses	88
3.3.4	Association of immuno-electrophoretically identified impurities with ACTH response inhibition	90
3.3.5	Conclusions concerning inhibitory factors	90
3.4	GENERAL CONCLUSIONS	93

CHAPTER 4	STRAIN DIFFERENCES IN ACTH- AND dbcAMP-STIMULATED STEROIDOGENESIS IN POOLED ADRENAL INCUBATIONS	95
4.1	INTRODUCTION	95
4.2	PHENOTYPE DEFINITION	96
4.2.1	ACTH dose response relationships	96
4.2.2	dbcAMP dose response relationships	104
4.2.3	Strain specific variation in corticosterone production rate with length of incubation	110
4.2.4	Conclusions concerning phenotype definition	114
4.3	STRAIN COMPARISONS IN ACTH-STIMULATED MAXIMAL STEROIDOGENESIS	115
4.3.1	BSA-inhibited pooled experiments	116
4.3.2	Uninhibited experiments using manually dissociated cells	117
4.3.3	Uninhibited experiments using cells dissociated by machine	118
4.3.4	A large experiment employing exceptionally large pools of adrenals that were machine-dissociated	118
4.3.5	Correction of machine dissociated experiments to make them comparable to manually dispersed ones	119
4.4	CONCLUSIONS	
CHAPTER 5	ACTH-STIMULATED STEROIDOGENESIS BY CELLS FROM SINGLE ANIMALS	128
5.1	INTRODUCTION	128
5.2	CORRECTION OF INHIBITED VALUES	129
5.3	STATISTICAL METHODS	133
5.3.1	Mann-Whitney U test	133
5.3.2	Kolmogorov-Smirnov two-sample test	134
5.3.3	F test	135

	<u>Page No.</u>
5.4 PARENTAL AND F_1 HYBRID STRAINS	136
5.4.1 Results	136
5.4.2 Statistical analyses	141
5.4.2.1 Normal values	144
5.4.2.2 All values	144
5.5 BACKCROSSES TO DBA	145
5.5.1 Results	145
5.5.2 Statistical analyses	145
5.5.2.1 Normal values	150
5.5.2.2 All values	150
5.6 BACKCROSSES TO C57	150
5.6.1 Results	150
5.6.2 Statistical analyses	154
5.6.2.1 Normal values	154
5.6.2.2 All values	154
5.7 BACKCROSSES INVOLVING CBA x DBA F_1 MICE	157
5.7.1 Results	157
5.7.2 Statistical analyses and conclusions	160
5.8 THE COMPONENTS OF INHERITANCE OF STEROID SYNTHETIC CAPACITY EXPRESSED IN SEGREGANT ADRENAL TYPES	161
5.8.1 Evidence of genetic control underlying phenotypic seg- regation	161
5.8.1.1 Comparison of variances	161
5.8.1.2 Segregation with genetic markers	163
5.8.1.2.1 Iso-citrate dehydrogenase (<u>Idh-1</u>) and malic enzyme (<u>Mod-1</u>)	163
5.8.1.2.2 Coat colour markers; <u>d</u> , <u>b</u> and <u>a</u>	167
5.8.2 Differences between recip- rocal crosses	170
5.8.2.1 X-chromosome linkage	171
5.8.2.2 Differences of apparently non- genetic origin	171

5.8.3	Evidence that only a few gene differences differentiate the DBA and C57 R-phenotypes	173
5.8.3.1	Differentiation of cumulative frequency distributions	174
5.8.3.2	Test for a single gene difference using original values	174
5.8.3.3	Reduction of maternal effect	178
5.8.3.4	Test for a single gene difference using values adjusted to minimise maternal effects	179
5.9	CONCLUSIONS	182
CHAPTER 6	STRAIN DIFFERENCES IN THYMUS, SPLEEN AND TESTIS WEIGHTS AND THEIR RELEVANCE TO ADRENAL FUNCTION	199
6.1	INTRODUCTION	199
6.2	STATISTICAL METHODS	206
6.2.1	General strategy	206
6.2.2	Non-parametric tests	207
6.2.3	Parametric statistics	209
6.2.4	Correction of spleen dry weights obtained under different drying conditions	209
6.3	CORRELATION OF ORGAN WEIGHTS AND CORTICOSTERONE PRODUCTION (R) WITH BODY WEIGHTS	211
6.4	STRAIN DIFFERENCES IN THYMUS WEIGHTS	218
6.4.1	Strain differences in thymus dry weight within and across 14-day age groups	218
6.4.1.1	Differences between strains in thymic involution	218
6.4.1.2	Thymus dry weight differences between strains within age groups	222
6.4.2	Comparisons of thymus dry weight variances	225
6.4.3	Thymus weights and involution trends associated with values selected for hetero- or homozygosity at the <u>Idh-1</u> , <u>Mod-1</u> , <u>d</u> , <u>b</u> and <u>a</u> genetic loci	229

Page No.

6.4.3.1	Marker-linked influences on thymic involution	229
6.4.3.2	Associations of marker gene allotypes with differences in thymus weight	235
6.5	STRAIN DIFFERENCES IN SPLEEN WEIGHT	239
6.5.1	Strain differences in spleen dry weight within, and across, 14-day age groups	239
6.5.1.1	Differences in spleen growth between strains	239
6.5.1.2	Spleen dry weight differences between strains within age groups	242
6.5.2	Comparisons of spleen dry weight variances	244
6.5.3	Spleen weights and growth trends associated with values selected for hetero- or homo-zygosity at the <u>Idh-1</u> , <u>Mod-1</u> , <u>d</u> , <u>b</u> and <u>a</u> genetic loci	247
6.5.3.1	Marker-linked influences on splenic weight change	247
6.5.3.2	Associations of marker gene allotypes with differences in spleen weight	251
6.6	STRAIN DIFFERENCES IN TESTIS WEIGHT	256
6.6.1	Strain differences in testis dry weight within, and across, 14-day age groups	256
6.6.1.1	Differences in testis growth between strains	256
6.6.1.1	Testis dry weight differences between strains within age groups	260
6.6.2	Comparisons of testis dry weight variances	265
6.6.3	Testis weights and growth trends associated with values selected for hetero- or homo-zygosity at the <u>Idh-1</u> , <u>Mod-1</u> , <u>d</u> , <u>b</u> and <u>a</u> genetic loci	268
6.6.3.1	Marker-linked influences on testis growth	268
6.6.3.2	Association of marker gene allotypes with differences in testis weight	275

6.7	STRAIN DIFFERENCES IN CORTICOSTERONE PRODUCTIVE CAPACITY (R) CORRELATION WITH AGE	278
6.7.1	Strain differences in age-re- lated changes in corticost- erone productive capacity (R)	278
6.7.2	Marker-linked influences on change in corticosterone productive capacity (R) with age	281
6.8	CORRELATIONS BETWEEN LYMPHORETICULAR TISSUE SIZE, TESTIS WEIGHT AND CORTICOSTERONE PRODUCTIVE CAPACITY (R)	283
6.8.1	Correlations present in strains unselected for genetic marker type	283
6.8.2	Difference in correlation associated with differences in marker gene allotype	285
6.8.2.1	Thymus absolute weight v. corticosterone productive capacity, R	294
6.8.2.2	Spleen absolute weight v. corticosterone productive capacity, R	295
6.8.2.3	Thymus absolute weight v. testis absolute weight	295
6.8.2.4	Spleen absolute weight v. testis absolute weight	296
6.8.2.5	Testis absolute weight v. corticosterone productive capacity, R	298
6.8.2.6	Thymus absolute weight v. spleen absolute weight	299
6.9	CONCLUSIONS	301
CHAPTER 7	GENERAL CONCLUSIONS	316
	BIBLIOGRAPHY	332

LIST OF TABLES

	<u>Page No.</u>
2.1 The relationship between corticosterone concentration and its fluorescence in acidic ethanol.	29
2.2 Mean fluorescences for different corticosterone concentrations.	30
2.3 Comparison of haemocytometer and Coulter counts.	39
2.4 Comparison of total cell yields, computed from cell counts performed at threshold setting = 21, for the original and the replacement Coulter counters.	42
2.5 Comparison of total steroid yields for uninhibited experiments before and after purity of BSA was controlled.	44
2.6 Responses to ACTH and dbcAMP in experiments using inhibitory and controlled BSA's, and also the original and replacement Coulter counters.	45
2.7 Ratio of counts at T = 2.5 to those at T = 21 for each strain.	50
2.8 t test probabilities of difference between strain specific cell count ratios.	50
3.1 Relationship of enzyme and calcium concentrations to cell yield and steroidogenic capacity.	72
3.2 Effect of collagenase concentration, volume and hyaluronidase on cell yield.	73
3.3 Effects of double collagenase incubation and hyaluronidase on cell yield and responsiveness.	74
3.4 Effects of including a ball-bearing during dispersion on cell yield, viability and responsiveness.	76
3.5 Comparison of cell yields, viabilities and responses using syringe needles and pipettes to dissociate cells.	77
3.6 Comparison of steroidogenic responses and the viabilities of cells dispersed and incubated using different BSA batches.	87

Page No.

3.7	Comparison of responses to ACTH and dbcAMP in experiments with inhibited ACTH responses.	89
3.8	Comparison of uninhibited ACTH with dbcAMP-induced responses.	89
4.1	Dose response to ACTH (Experiment 3).	97
4.2	Dose response to ACTH (Experiment 6).	98
4.3	Dose response to ACTH (Experiment 7).	99
4.4	Dose responses of DBA and CBA female cells.	99
4.5	Dose responses of cells from DBA, $F_1(N)$ and C57 males.	100
4.6	Dose responses of CBA and $F_1(Q)$ male cells.	101
4.7	Dose responses of C57, DBA and $F_1(N)$ male cells to dbcAMP, and their maximal responses to ACTH.	105
4.8	Maximal responses of C57, DBA and $F_1(N)$ male cells to dbcAMP and ACTH.	107
4.9	Responses of DBA and $F_1(N)$ male cells to dbcAMP.	108
4.10	Response of CBA male cells to dbcAMP (Experiment 62).	109
4.11	Time course of corticosterone production by DBA, $F_1(N)$ and C57 male cells in response to ACTH.	110
4.12	Strain specific changes in steroidogenesis with time. Slope; (rate of corticosterone synthesis over specified time period).	112
4.13	Rates of decline of corticosterone production rates.	113
4.14	Probable effects on corticosterone production rates of time-dependent and time-independent sub-phenotypes.	114
4.15	Corticosterone production (R).	116
4.16	Corticosterone production (R)	117

	<u>Page No.</u>
4.17	Corticosterone production (R). 118
4.18	Corticosterone production (R). 119
4.19	Corticosterone production (R). 120
4.20	Comparison between corticosterone production by whole adrenals and cell suspensions (used in the present work). 126
5.1	Correction factors; consisting of the ratio of normal (mean of all uninhibited data) to inhibited steroid production, for each strain and experiment. 130
5.2	Grouping of correction factors from experiments assumed to have been inhibited by similar amounts of BSA impurity. 132
5.3	Parental Strain: DBA/2J. 137
5.4	Parental Strains: C57BL/Tb and CBA/FaCam. 138
5.5	F ₁ Hybrids between DBA and C57 (dams of crosses given first). 139
5.6	F ₁ Hybrid (Q): CBA dam x DBA Sire. 140
5.7	Probabilities ($\times 10^2$) of parental and F ₁ Mann-Whitney and Kolmogorov-Smirnov single-tailed tests. 143
5.8	Backcross OD (O dam x DBA). 146
5.9	Backcross ND (N dam x DBA). 147
5.10	Single-tailed probabilities of Mann-Whitney and Kolmogorov-Smirnov tests on OD, ND and parental strains. 149
5.11	Backcross OC (O dam x C57). 151
5.12	Backcross NC (N dam x C57). 152
5.13	Backcross CN (C57 dam x N). 153
5.14	Single-tailed probabilities ($\times 10^2$) of Mann-Whitney and Kolmogorov-Smirnov tests on NC, CN, OC and parental lines. 156

	<u>Page No.</u>
5.15 Backcross QD (Q dam x DBA).	158
5.16 Backcross QB (Q dam x CBA).	158
5.17 F tests on variances of normal and all values.	162
5.18 Single-tailed probabilities of Mann-Whitney and Kolmogorov-Smirnov tests of differences in R associated with <u>Idh-1</u> type.	166
5.19 Means, standard deviations and coefficients of variation (V) for reciprocal crosses.	170
5.20 Probabilities of backcross distributions fulfilling a single gene model.	175
5.21 R values of individual male DBA and C57 decedents.	186
5.22 Mann-Whitney single-tailed probabilities for tests between all stimulated and all repressed data.	186
5.23 R values of individual virgin CBA females and of pooled DBA and C57 female adrenals.	189
5.24 Adrenal colour, <u>Idh-1</u> type and corticosterone production (R).	193
6.1 Regression statistics used to correct overheated spleen weights.	210
6.2 Non-parametric correlation coefficients for absolute organ weight versus body weight (BWT).	212
6.3 Parametric correlation coefficients for absolute organ weight versus body weight (BWT).	213
6.4 Partial correlation coefficients (parametric) between absolute organ weight and body weight (BWT) controlling for age.	214
6.5 Partial correlation coefficients (parametric) between absolute testis weight (testisa) or corticosterone productivity (R) and body weight (BWT), controlling for age, and either R or testis, respectively.	215
6.6 Mean absolute thymus dry weights for different strains and age groups.	220

		<u>Page No.</u>
6.7	Mean relative thymus dry weights for different strains and age groups.	220
6.8	Probabilities (%'s) for Friedman two-way analyses of variance between absolute thymus weight means of different age groups and strains.	221
6.9	Kruskal-Wallis one-way analysis of variance of absolute and relative thymus dry weights for combinations of strains for each age group.	223
6.10	Mann-Whitney two-tailed probabilities of tests between pairs of strains within each age group.	223
6.11	Absolute thymus dry weight variances ($\times 10^2$) for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.	226
6.12	Relative thymus dry weight variances ($\times 10$) for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.	226
6.13	Variance ratios for absolute and relative thymus dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.	228
6.14	Mean absolute thymus dry weights for different <u>Idh-1</u> types and age groups.	230
6.15	Mean relative thymus dry weights for different <u>Idh-1</u> types and age groups.	230
6.16	Mean absolute thymus dry weights for different <u>Mod-1</u> types and age groups.	231
6.17	Mean relative thymus dry weights for different <u>Mod-1</u> types and age groups.	231
6.18	Mean absolute thymus dry weights for different <u>d</u> types and age groups.	232
6.19	Mean relative thymus dry weights for different <u>d</u> types and age groups.	232
6.20	Mean absolute thymus dry weights for different <u>b</u> types and age groups.	234
6.21	Mean relative thymus dry weights for different <u>b</u> types and age groups.	234

Page No.

6.22	Mean absolute thymus dry weights for different <u>a</u> types and ages.	234
6.23	Mean relative thymus dry weights for different <u>a</u> types and ages.	234
6.24	Absolute thymus dry weight means for different marker types of individual and combined strains using values from all ages.	236
6.25	Relative thymus dry weight means for different marker types of individual and combined strains using values from all ages.	237
6.26	Mann-Whitney two-tailed probabilities for tests between NC and OC thymus weights of the same marker gene allotype.	238
6.27	Mean absolute spleen dry weights for different strains and age groups.	240
6.28	Mean relative spleen dry weights for different strains and age groups.	241
6.29	Kruskal-Wallis one-way analysis of variance of absolute and relative spleen dry weights for combinations of strains for each age group.	243
6.30	Mann-Whitney two-tailed probabilities for tests between pairs of strains within each age group.	243
6.31	Absolute spleen dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.	245
6.32	Relative spleen dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.	245
6.33	Variance ratios for absolute and relative spleen dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.	246
6.34	Mean absolute dry spleen weights for different <u>Idh-1</u> types and age groups.	248

Page No.

6.35	Mean relative dry spleen weights for different <u>Idh-1</u> types and age groups.	248
6.36	Mean absolute dry spleen weights for different <u>Mod-1</u> types and age groups.	249
6.37	Mean relative dry spleen weights for different <u>Mod-1</u> types and age groups.	249
6.38	Mean absolute spleen dry weights for different <u>d</u> types and age groups.	250
6.39	Mean relative dry spleen weights for different <u>d</u> types and age groups.	250
6.40	Mean absolute uncorrected spleen dry weights for different <u>b</u> types and age groups.	252
6.41	Mean relative uncorrected spleen dry weights for different <u>b</u> types and age groups.	252
6.42	Mean absolute uncorrected spleen dry weights for different <u>a</u> types and age groups.	252
6.43	Mean relative uncorrected spleen dry weights for different <u>a</u> types and age groups.	252
6.44	Mann-Whitney two-tailed tests between mean absolute and relative spleen dry weights selected for different marker allotypes.	253
6.45	Mann-Whitney two-tailed probabilities for tests between OD and ND spleen weights of the same marker gene allotype.	254
6.46	Mean absolute testis dry weights for different strains and age groups.	257
6.47	Mean relative testis dry weights for different strains and age groups.	257
6.48	Probabilities (%'s) for Friedman two-way analyses of variance between absolute and relative testis weight means of different age groups and strains.	259
6.49	Kruskal-Wallis one-way analysis of variance of absolute and relative testis dry weights for combinations of strains for each age group.	261

Page No.

6.50	Mann-Whitney two-tailed probabilities for tests between testis weights of pairs of strains within each age group.	263
6.51	Mean body weights for different strains and age groups.	264
6.52	Absolute testis dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group mean.	266
6.53	Relative testis dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group mean.	266
6.54	Variance ratios for absolute and relative testis dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.	267
6.55	Mean absolute testis weights for different <u>Idh-1</u> types and age groups.	269
6.56	Mean relative testis weights for different <u>Idh-1</u> types and age groups.	269
6.57	Mean absolute testis weights for different <u>Mod-1</u> types and age groups.	271
6.58	Mean relative testis weights for different <u>Mod-1</u> types and age groups.	271
6.59	Mean absolute testis dry weights for different <u>d</u> types and age groups.	272
6.60	Mean relative testis dry weights for different <u>d</u> types and age groups.	272
6.61	Mean absolute testis dry weights for different <u>b</u> types and age groups.	273
6.62	Mean relative testis dry weights for different <u>b</u> types and age groups.	273
6.63	Mean absolute testis dry weights for different <u>a</u> types and age groups.	273
6.64	Mean relative testis dry weights for different <u>a</u> types and age groups.	273

Page No.

6.65	Mann-Whitney two-tailed probabilities for tests between reciprocal cross absolute testis weights of the same marker type.	276
6.66	Mean corticosterone productive capacities (R's) for different strains and age groups.	280
6.67	Mean corticosterone productive capacities (R's) for different <u>Idh-1</u> types and age groups.	280
6.68	Mean corticosterone productive capacities (R's) for different <u>b</u> types and age groups.	282
6.69	Mean corticosterone productive capacities (R's) for different <u>a</u> types and age groups.	282
6.70	Correlations between absolute organ weights for each strain.	284
6.71	Zero order and partial correlation coefficients for thymus absolute weight v. corticosterone productive capacity (R) for different marker types.	287
6.72	Zero order and partial correlation coefficients for spleen absolute weight v. corticosterone productive capacity (R) for different marker types.	288
6.73	Zero order and partial correlation coefficients for thymus absolute weight v. testis absolute weight for different marker types.	289
6.74	Zero order and partial correlation coefficients for spleen absolute weight v. testis absolute weight for different marker types.	290
6.75	Zero order and partial correlation coefficients for testis absolute weight v. corticosterone productive capacity (R) for different marker types.	291
6.76	Zero order and partial correlation coefficients for thymus absolute weight v. spleen absolute weight for different marker types.	292
6.77	Frequency distributions of death dates of 'suppressed' and 'stimulated' generations of DBA and C57 mice.	311

Page No.

6.78	Mean thymus and testis absolute dry weights from DBA and C57 mice of alternate generations.	312
6.79	Correlation coefficients for DBA and C57 variables pooled from alternating generations.	314

LIST OF FIGURES

		<u>Page No.</u>
1.1	Components of a plant endocrine response	1
2.1	Linear relationship between corticosterone concentration and fluorescence in acidic ethanol.	31
2.2	CBA female adrenal cell Coulter count versus red threshold setting.	36
2.3	Combined C57 male adrenal Coulter counts versus red threshold setting.	38
3.1	Principal components of dissociating machine with Meccano parts used shown in brackets (air expulsion cycle nearly complete).	80
4.1	Dose response curves of DBA, C57 and $F_1(N)$ male adrenal cells to ACTH.	102
4.2	Dose response curves of DBA, CBA and $F_1(Q)$ male adrenal cells to ACTH.	103
4.3	Pooled cell responses to various doses of dbcAMP and to maximal stimulation by ACTH.	106
4.4	Interaction of incubation period length with strain-specific corticosterone production.	111
5.1	The distributions of R for individual members of parental and F_1 hybrid strains.	142
5.2	The distributions of R for individual members of the backcrosses (N dam x DBA) and (O dam x DBA). (DBA and F_1 distributions are shown for the sake of comparison).	148
5.3	R values for individual members of the backcrosses (N dam x C57), (C57 dam x N) and (O dam x C57) (C57 and F_1 distributions are shown for the sake of comparison).	155
5.4	R values of individual members of the backcrosses (Q dam x DBA) and (Q dam x CBA). (Parental and F_1 distributions are shown for the sake of comparison).	159
5.5	<u>Idh-1</u> segregation and steroidogenic capacity (R) using uncorrected values only.	164

Page No.

5.6	<u>Idh-1</u> segregation and steroidogenic capacity (R) using corrected and uncorrected values.	165
5.7	Brown coat colour (<u>b</u>) segregation and steroidogenic capacity (R) using corrected and uncorrected values.	169
5.8	Cumulative frequency distributions of NC, CN and OC (all data).	172
5.9	Backcrosses to C57; OC and NC with one-gene composite distributions; 50% C57:50% F_1 .	176
5.10	Backcrosses to DBA; OD and ND with one-gene composite distributions; 50% DBA:50% F_1 .	177
5.11	50% OC:50% NC and the one-gene composite distribution; 50% C57:25% N:25% O, both corrected for maternal influence.	180
5.12	50% OD:50% ND and the one-gene composite distribution; 50% DBA:25% N:25% O, both corrected for maternal influence.	181
5.13	Cumulative frequency distributions of ND and OD (all values).	195
6.1	The regression lines of absolute testis dry weight on absolute spleen dry weight for backcross QD mice of different <u>d</u> allotypes.	297

PLATE

3.1	Immuno-electrophoresis of three different batches of BSA.	91
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LIST OF ABBREVIATIONS

ald	adrenal lipid depletion
ACTH	adrenocorticotrophic hormone
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate or adenosine-3',5'-monophosphate
dbcAMP	dibutyryl-adenosine-3',5'-monophosphate
DNase	deoxyribonuclease
Id	<u>Idh-1</u> , the genetic locus controlling murine isocitrate dehydrogenase
KRBG	Krebs Ringer bicarbonate buffer containing 0.2% glucose
KRBG-BSA	KRBG containing 4% bovine serum albumin
K-S 1(or 2)-tail test	Kolmogorov-Smirnov single (or double)-tailed test
K-W test	Kruskal-Wallis one-way analysis of variance
Mod	<u>Mod-1</u> , the genetic locus controlling murine malic enzyme
M-W 1(or 2)-tail test	Mann-Whitney single (or double)-tailed U test
NADP	nicotinamide adenosine diphosphate
NPAR	non-parametric
P	statistical probability expressed as proportions of unity in text, and as %'s (or %'s x 10 ²) in tables, where specified
PAR	parametric
PART. CORR.	partial correlation coefficient
R	pgms. corticosterone produced per viable cell per hour in response to 2.5 mU ACTH per ml incubation medium
r(NPAR)	Spearman rank correlation coefficient
r(PAR)	Pearson product-moment correlation coefficient
T	Coulter counter threshold setting

ABSTRACT

Relatively simple genetic models have been thought to explain differences between populations of man and rodent with respect to some endocrine functions. In the case of resting plasma corticosterone levels, differences between two mouse strains have been accounted for by a two-gene model. Genetic analysis of adrenal corticosteroidogenesis in the mouse, particularly in strains carrying ald (adrenal lipid depletion) genes, has been limited by the use of relatively insensitive techniques. In the present study, the in vitro corticosterone productive capacities of adrenals from 7 - 17 week old, male, DBA/2J, CBA/FaCam, C57BL/Tb and hybrid mice were measured using a specially modified existing rat isolated adrenal cell technique, permitting greater sensitivity of measurement to be achieved compared with previously used mouse adrenal slice techniques. Inter-experimental repeatability was high, except in cases where inhibition of steroidogenesis occurred due to the use of certain batches of bovine serum albumin (BSA). This protein was used in incubation media and it was found that batches of BSA with the greater amounts of protein impurities inhibited steroidogenesis the most. Although such inhibition was observed to affect ACTH responsiveness, it was absent when steroidogenesis was stimulated with dbcAMP, suggesting inhibition was exerted extra-cellularly. Corrections were made, on the basis of discernible degrees of inhibition by different batches of BSA, to provide estimates of steroidogenesis where experiments had been performed using inhibiting batches of serum albumin.

Using cell suspensions prepared from adrenals pooled from genetically uniform animals, dose response studies indicated that a concentration of 2.5 mU ACTH/ml incubation medium was likely to produce maximal steroidogenic responses in all genotypes. A time-course study revealed that under conditions of maximal ACTH stimulation, cells of strain DBA/2J, which carry a gene causing adrenal lipid depletion, produced markedly less corticosterone with time compared with C57BL/Tb cells. It was speculated, from F_1 (C57* x DBA) time-course values, that an interaction between time-dependent and time-independent characters might exist in this hybrid, signifying the existence of at least a two-gene difference between parental types.

Pooled DBA/2J cells, unlike those of strain C57BL/Tb, possessed significantly reduced responses (about -10%) to ACTH compared to dbcAMP, indicating a relative inefficiency of the ACTH response in this strain.

Means for the specified phenotype 'R' (pgms corticosterone produced/viable cell/hour in response to 2.5 mU/ml ACTH) were measured, using a number of pooled adrenal experiments, for parental and F_1 hybrid lines. Significant differences in mean R values were observed; strains C57BL/Tb and CBA/FaCam possessing mean values that were approximately 2.5X and 2X, respectively, the mean DBA/2J R value. The F_1 (C57 x DBA) possessed a mean R value that was significantly different from either parental type, whereas the F_1 (CBA x DBA) value was indistinguishable from that of strain CBA/CamFa.

* strain of dam given first in hybrid crosses.

Investigation of corticosterone production by cells derived from individual adrenal pairs confirmed the strain differences observed for pooled organs and indicated the presence of stimulated and repressed classes of adrenal functional state within genetically uniform strains. The significantly greater (+30%) mean R value of the F_1 (DBA x C57), compared with that of its reciprocal cross F_1 (C57 x DBA), could not be accounted for by sex-chromosome linkage. The significantly greater (+15%) mean R values of backcrosses with F_1 (C57 x DBA) mothers, compared with those of reciprocal crosses with F_1 (DBA x C57) mothers, confirmed the operation of a negative maternal effect. Backcross progeny for the strains studied showed more variance in their R values than those of parental lines, although, probably due to small sample sizes, only one backcross displayed a significantly increased variance. When data from backcrosses between strains DBA/2J and C57BL/Tb were tested against theoretical distributions expected for a one-gene difference, only one out of four backcrosses showed discordance. In the same crosses, the C57 allele of the Idh-1 locus was significantly associated with higher R value.

When sample sizes were increased, by inclusion of corrected inhibited R values, all the above results were strengthened. In addition to greater evidence of significantly increased backcross variances and departure from a one-gene model (even after attempted removal of maternal effects), the C57 allele of the b locus appeared to be linked with higher mean R value.

It was speculated, on the basis of observations on adrenal lipid content, that R value was affected by the Idh-1-linked factor, perhaps,

by an alteration in stores of lipid precursors. This, and possibly the b-linked effect, appeared to be modified in their expressions by the maternal influence.

Although no evidence of genetic segregation between strains was observed for spleen weight, another lymphoreticular target for corticosteroids, the thymus, displayed significant genetic effects with respect to size. The DBA alleles of the Idh-1 and Mod-1 loci were associated with lighter (-20%) and heavier (+34%) thymus weight, respectively. An argument was made that the low thymus weights of mice with DBA Idh-1 allotypes appeared to be co-adapted to their low adrenal corticosterone productive capacities.

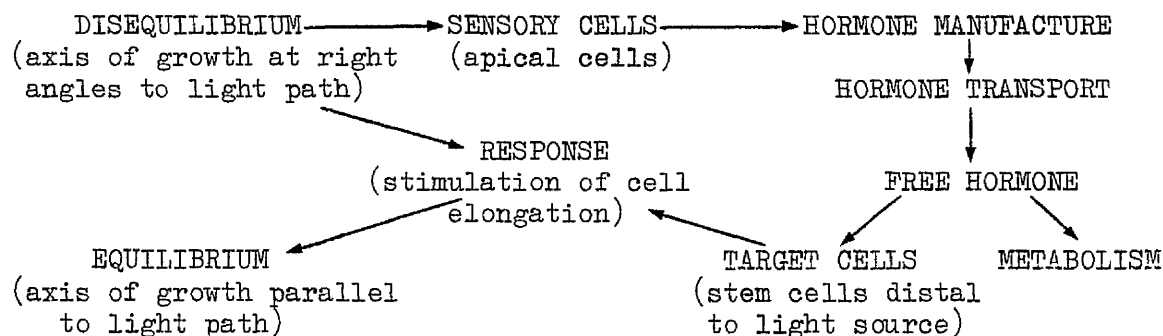
It was speculated, with reference to the relative inefficiency of the DBA/2J response to ACTH (compared with dbcAMP) and the positive partial correlations found between thymus weight and R value in mice homozygous for the DBA allele of the Idh-1 locus, that the Idh-1-linked gene may act by altering brain and thymus sensitivity to corticosterone. On the basis of this locus in fact being an ald gene, it was suggested that this provided support for previous hypotheses that had suggested ald genes had extra-adrenal primary sites of action.

CHAPTER 1

INTRODUCTION

Although morphological innovation is frequently regarded as the corner-stone of Darwinian evolution, such a view belies the, albeit covert, importance of genetic control mechanisms, without whose exponential development, structural improvements could not have occurred. Thus, although the lower eukaryote, Aspergillus nidulans, leads a similar saprophytic existence to many prokaryotes, its co-ordinated induction of genes with related functions, which are often dispersed throughout the genome (Pateman and Kinghorn, 1976), is many times more complex than that of bacteria, which relies on the physical contiguity of genes with related functions (Jacob and Monod, 1961). Increased specialisation of function required that multicellular eukaryotes such as plants developed rudimentary endocrine systems to co-ordinate development and adaptive responses; the components of which are shown in Figure 1.1.

Figure 1.1 Components of a plant endocrine response.



Genetic variants have been isolated for such endocrine systems in plants that affect development in the moss, Physcomitrella patens (Cove and Ashton, 1975), and result in gibberellic acid insensitivity in wheat (Gale and Marshall, 1973).

The potential offered by endocrine control was exploited to the full, however, with the organogenesis in animals that resulted from the need to trap, digest and assimilate food. Segregation of DNA, in association with nuclear proteins, within a nuclear envelope, enabled two major categories of response to hormones to evolve; specific gene activation, usually as part of a profound adaptive or developmental change, and a more general stimulation of messenger RNA translation and enzyme activity, in response to sudden, and usually more transient, adaptive needs.

The former type of response is evoked by cell-permeable hormones such as steroids and thyronines which have been observed to become concentrated in target cell nuclei (Clark et al., 1973; Samuels and Tsai, 1973). Visual evidence of gene induction by the steroid, ecdysone, was obtained from insect salivary gland chromosomes (Clever and Karlson, 1960). The biochemistry of gene induction is, however, complex with apparently many nuclear components being involved (Marver et al., 1972; Schrader et al., 1972; Yamamoto et al., 1974). Non-histone proteins have been shown to be essential for binding between chromatin and hormone-receptor complexes (Spelsberg et al., 1971) and perhaps for locating hormone on specific regions of deoxyribonucleic acid (King and Mainwaring, 1974).

The different actions of aldosterone in two strains of mouse have been associated with different types of nuclear binding of the hormone in kidney cells (Stewart, 1975).

Another type of pre-translational control resulting in altered apparent gene activity has been thought to consist of hormonal inhibition of messenger ribonucleic acid degradation (Tomkins et al., 1969).

The other class of hormones, those that tend to remain outside the cell membrane, such as peptides and catecholamines, exert their largely extra-nuclear control by first becoming attached to membrane-bound receptors (Rall et al., 1957; Pastan et al., 1966). Occupied receptors, having become allosterically transformed, activate a variety of proteins, the most notable being adenylate cyclase. This enzyme was found to convert 5'-adenosine monophosphate to adenosine-3',5'-monophosphate (cyclic AMP or cAMP) (Robison et al., 1968) which, by the activation of a protein kinase, was responsible for the glycogen breakdown response to epinephrine (Walsh et al., 1968). Glucagon receptor numbers (Mirel et al., 1978) and ACTH receptor specificity (Schorr and Ney, 1971) have both been demonstrated to be deficient in rodent tumour cell lines and a mouse adrenocortical tumour cell line has been selected that appears to be insensitive to cAMP (Schimmer et al., 1977).

Phosphorylation of a variety of proteins, by means of cAMP-dependent protein kinases (Kuo and Greengard, 1969), results in altered cell membrane ion transport (Rudolph and Greengard, 1974), pre-existing enzyme activation (Soderling and Park, 1974) and

modulation of translation (Blat and Loeb, 1971; Wicks, 1974). Guanine-3', 5'-monophosphate (cGMP) has also been implicated as a 'second messenger' and appears to complement cAMP in this role (Goldberg et al., 1974) especially in producing opposing responses to hormones such as insulin and glucagon. The steroidogenic response of the adrenal cortex to ACTH has been shown to result from stimulation of early processes, such as the conversion of cholesterol to pregnenolone (Stone and Hechter, 1954; Koritz and Kumar, 1970), as well as later metabolic steps, especially those requiring 11-beta-hydroxylation (McKenna et al., 1979). Strains differences concerning later hydroxylation steps have been observed in mice (Badr and Spickett, 1965; Badr, 1970) and rats (Rapp and Dahl, 1972; Rapp and Dahl, 1976) that have been related to single gene effects.

Some functional overlap exists between these two major types of response as glucagon has been shown to affect transcription by histone phosphorylation in liver cells (Watson and Langan, 1973) and some steroids can exert effects on enzyme activity (Griffin et al., 1973) and cell membrane permeability (Ui and Mueller, 1963). Although maximal responses to peptide hormones have been observed in the absence of appreciable rises in cAMP concentrations (Nakamura et al., 1972; Mendelson et al., 1975), it has been suggested that intracellular translocation of undetectably small pools of cAMP could account for this phenomenon, as well as for the multiple responses induced by, particularly, trophic hormones (Catt and Dufau, 1977).

Evolution of the complex hierarchical power structures found in the endocrine systems of higher animals probably resulted from

selection, within potentially dominant glands, for production of specific metabolites that were rate-limiting with respect to hormone production by subordinate glands (Bellamy, 1971). Modification of target tissue responses to pre-existing hormones, observed as a general feature of endocrine evolution (Barrington, 1968), could also have assisted in the emergence of dominance. It has been hypothesised, with particular reference to the, effectively, lethal effects of sex determination mutants, such as testicular feminisation, that selection for increased genetic simplicity probably existed, in the course of endocrine evolution, in response to increasing mutation loads (Ohno, 1971). The multiplicity of effects controlled by cAMP probably represent an example of such genetic economy. Differences between mouse and rat inbred strains with respect to endocrine functions have been found, frequently, to be the result of single gene effects (Shire, 1979c). The basic feed-back cycles comprising a mammalian endocrine response are identical to the simple response loop of a plant shown in Figure 1.1; greater complexity being derived from the superimposition of many such loops in which endocrine organs are, themselves, target organs to more dominant glands, particularly the anterior pituitary, which in turn is ultimately governed by hypothalamic secretions. The extensive information that has accumulated, concerning pathological variants, in humans and rodents, indicates that every 'link' in the 'chain' of response is susceptible to genetic variation. The failure of pituitary gonadotrophin release in hpg/hpg mice is thought to be caused by a lesion in the hypothalamus (Cattanach et al., 1977).

Oestrogen binding to this part of the brain has been shown to be defective in mice homozygous for a p-sterile mutant allele (Johnson and Hunt, 1975). Genetically determined pituitary aplasia and growth hormone deficiency are known in man (Rimoin and Schimke, 1971; Laron, 1978) and the latter defect exists in dw/dw and df/df mice (Snell, 1929; Carsner and Rennels, 1960; Schaible and Gowen, 1961). Unresponsiveness to pituitary hormones have been shown to be genetically determined in cases of adrenal insensitivity to ACTH in man (Kershner et al., 1972), and peripheral unresponsiveness to growth hormone occurs in pg/pg mice (Rimoin and Richmond, 1972). The endocrine gland targets of trophic hormones have been shown to have heritable enzymic lesions producing altered hormone synthetic capacities. In man, thyroid hormone defects of a heritable nature exist (Stanbury, 1972; Shire, 1979b), and lesions in steroidogenic pathways have been found, such as a 17- α -hydroxylase defect expressed in both adrenal and gonad (Biglieri and Mantero, 1973). The vet locus in rats is associated with defective androgen production (Bardin et al., 1973) and adrenal hyperplasia occurs at puberty in lh/lh mice (Dung and Swigart, 1971). Genetic variants in target organ sensitivity have been observed with respect to thyroxine (Scheider et al., 1975) and testosterone (Meyer et al., 1975) in man. The latter, X-linked, locus (Tfm) has a similarly linked analogue in mice (Lyons and Hawkes, 1970; Bullock, 1979).

The occurrence of genetic variation at apparently all levels of the endocrine power structure indicates the evolutionary possibility of similar, nett, endocrine responses being achieved by

different combinations of variants for subsidiary functions, provided they tended to counter-balance each other's extreme effects. The evolution of such balanced combinations of polygenes (Mather, 1943) would be stimulated by the direct association of such combinations with increased viability, as well as the more general advantage of greater covert genetic flexibility (Thoday, 1953). It is thus thought that such co-adapted gene complexes, consisting of loci coding for key components of a response such as hormone production and target organ sensitivity, may be preserved by linkage in out-breeding populations, although, in theory, when genetic segregation is severely curtailed, these genes could reside on different chromosomes (Thoday, 1964; Shire, 1969b; Shire, 1974). This principle could be extrapolated to predict that the control of a balanced combination, such as high hormone productive capacity with low target organ sensitivity, by a single gene, would be of even greater advantage in out-breeding populations.

Evidence of co-adaptive evolution, by its very nature, can only be observed in normal individuals. Ideally, the individual components of a hormone response should be examined, separately, under genetically and environmentally controlled conditions. Although true-stock Africans have been found to possess smaller adrenal cortices (Allbrook, 1956) but larger plasma cortisol levels (Briggs and Briggs, 1972) than non-Africans, most studies on non-pathological endocrine variation have used inbred strains of animals, especially mice, which have been studied extensively, already, and which comply with the above strictures, perfectly.

Some general examples of co-adaptive evolution have already been obtained for the latter species. Kidneys of CBA, and a wild stock, of relatively recent establishment (Spickett et al., 1967), Peru, were found to be specifically adapted to respond to the vaso-pressin molecule peculiar to each strain (Stewart, 1973; Stewart, 1979), and also to be capable of regulating sodium ion balance to similar extents, but by contrasting dependencies on aldosterone (Stewart et al., 1972; Stewart, 1975), which may be linked, in some way, to differences in adrenal zona glomerulosa development associated with ACTH plasma levels (Shire and Spickett, 1968a; Shire and Stewart, 1972), although aldosterone appears to be produced to similar extents in these strains (Papaioannou and Fraser, 1974).

In another case, C57BL/10J mice were found to possess small testes and to be androgen deficient (Shire and Bartke, 1972). Reproductive success was assured in this strain, however, by the co-adaptation of heightened seminal vesicle sensitivity to androgen (Bartke, 1974).

Indirect evidence, from stress-induced eosinopenia, suggested strain differences, with respect to mouse corticosterone synthesis (Wragg and Speirs, 1952; Thiessen and Nealey, 1962). Resting levels and plasma concentrations of corticosterone, following stress, or ACTH injection, were found to be different for a number of strains of mice, although, probably as a result of balanced co-adaptation with respect to steroid synthesis and degradation, differences in the former levels were much smaller (Levine and Treiman, 1964; Kakihana et al., 1968; Treiman et al., 1970; Eleftheriou and Bailey, 1972; Hawkins et al., 1975; Redgate and Eleftheriou, 1975). One of the studies, using inbred lines derived from

segregants between BALB/C and C57BL/6 strains suggested that two gene differences could account for the higher corticosterone resting level of the former strain (Eleftheriou and Bailey, 1972). The upper extreme of corticosterone production, thus, appeared to be exemplified by BALB/c mice, while another strain, AKR/O, seemed to occupy the opposite pole as it revealed very low resting and stressed plasma corticosterone levels (Levine and Treiman, 1964; Redgate and Eleftheriou, 1975).

The exceptional nature of AKR adrenal function was noted originally in a search for the cause of the high incidence of spontaneous leukaemia that afflicts this strain. The lipid vesicles containing adrenal cholesterol esters were found to be greatly reduced in a way that appeared to depend on the androgen surge at puberty, in males, and its interaction with a single gene effect (Arnesen, 1956; Molne, 1969c). The effect of adrenal lipid depletion on the development of the particularly severe form of leukaemia, found in AKR's, was eventually thought to be, at best, permissive, as the adrenal trait was found in crosses between AKR/O and WLO strains, accompanied by reduced incidence of leukaemia (Arnesen, 1974). In fact, the reduced corticosterone synthesis by adrenal bisects from adult male mice, homozygous for the ald gene, in response to ACTH (Solem, 1967), appeared to be compensated for by increased sensitivity of the thymus, in AKR's at any rate, to corticosterone (Metcalf, 1960). The cause of lipid-depletion in animals homozygous for the ald gene remains a mystery. It was originally found that prolonged treatment with high doses of ACTH could restore adrenal lipid levels to normal (Arnesen, 1956); a

view that was abandoned when lipid-depleted adrenals appeared to be hyperfunctional, histologically (Molbert and Arnesen, 1960), and cortisone administration was found to allow lipid re-accumulation (Arnesen, 1964). This evidence, suggesting heightened pituitary secretion of ACTH in ald/ald mice, was supported by experiments involving hypophysectomy and ACTH administration (Brabrand and Molne, 1968). A comparison of the effects of dexamethasone and ACTH treatment on adrenal weights, of intact AC (ald/ald) and C57BL mice, however, was interpreted as indicating the absence of any pituitary abnormality in lipid-depleted animals (Molne, 1969a).

Meanwhile, the first serious genetic analyses were being undertaken with respect to adrenal structure and development (Shire, 1965a; Shire, 1965b; Shire and Spickett, 1967; Shire and Spickett, 1968a; Shire and Spickett, 1968b) as well as corticosteroid production (Badr and Spickett, 1965b); single gene differences between strains being found in both these areas. Progress in this field (Dunn, 1970; Shire, 1974; Shire, 1979a; Shire, 1981) as well as more general aspects of endocrine genetics (Shire, 1976; Shire, 1979d), have been reviewed, regularly.

As strain DBA/2 had been shown to possess a corticosterone plasma stress response of similar magnitude to that of strain AKR (Levine and Treiman, 1964; Treiman et al., 1970) their adrenals were examined, and found to display an identical lipid phenotype to AKR (Doering et al., 1972; Doering et al., 1973). Lipid loss, as with the AKR syndrome, was androgen-dependent, was found to affect cholesterol ester, rather than free cholesterol, levels, and appeared to exert a rate-limiting effect on corticosterone production in

genetically uniform animals (Doering et al., 1972; Doering et al., 1973). Correlations of corticosterone production, from whole adrenals, with their cholesterol ester content was observed to break down in segregants between DBA/2 and C57BL/10; suggesting at least two genes probably accounted for the difference in corticosterone productive capacities of the two strains. In the same set of experiments, the retrieval of adrenals with lipid levels intermediate to those of DBA/2 and C57BL/10, from a backcross, to AC, of female F_1 (AC x DBA) mice, was interpreted as evidence of the non-allelism of the gene causing lipid depletion in DBA/2, designated ald-2, with that causing the syndrome in strains derived from AKR (Doering et al., 1973); an hypothesis that rested on the unproven premise that the site of action of ald genes was mainly intra-adrenal. Evidence from adrenal transplantation (Taylor et al., 1974) and chimaera (Shire, 1979a) studies, however, would suggest that the AKR-derived gene exerts its influence from a site outwith the adrenal cortex. Segregation of genes controlling other factors, such as testis function or androgen sensitivity, could have modified the expression of the ald phenotype, causing it to become separated into two phenotypic classes, in the segregants between AC and DBA.

Parallel studies on liver corticosterone catabolism indicated that C57BL strains and DBA/2 were co-adapted with respect to their adrenal hormone synthetic capacities, as the former strains appeared to metabolise corticosterone at a faster rate (Lindberg et al., 1972; Shire et al., 1972); thus accounting for the similar resting plasma corticosterone levels found for DBA/2 and C57BL mice (Levine and Treiman, 1964; Doering et al., 1972; Redgate and Eleftheriou, 1975).

Strain differences in adrenal corticosteroidogenesis have been observed for mice (Badr and Spickett, 1965b; Solem, 1967; Nandi et al., 1967; Badr, 1970; Doering et al., 1972; Doering et al., 1973) and for rats (Whitehouse and Vinson, 1976; Vinson et al., 1978), using either whole or fragmented adrenals. The inefficient and, possibly, artificial nature of this technique, compared with one using adrenal cells, dispersed using a collagenase technique (originated by Kloppenborg et al., 1968), was revealed when Wistar rat adrenal corticosteroidogenesis was measured using both techniques (Whitehouse and Vinson, 1976). When minced adrenals were used, ratios of ACTH-stimulated to basal (unstimulated) levels varied between 2 and 4 for males, whereas isolated cells, from the adrenals of similar animals, gave ratios of between 6 and about 10. The isolated cell technique gave stimulated:basal ratios that were of similar magnitude to those observed in mice for plasma corticosterone rises after stress (Levine and Treiman, 1964). The greater sensitivity of the isolated cell technique, which is, undoubtedly, due, in main part, to greater accessibility of cells to ACTH stimulation, possesses the additional advantage that shorter incubation times lessen any uncontrolled effects due to local accumulation of nascent steroid. As far as can be discerned, no previous attempts have been made to apply this more sensitive method of measuring corticosteroidogenic capacity to the study of mouse adrenal function. A major aim of this thesis was to develop a scaled-down version of an existing technique described for rat adrenal cell dispersion (Barofsky et al., 1973), that would enable isolated mouse cells to be studied. After the chapter describing

general methodology, a separate chapter is devoted to describing the history of the isolated cell technique, and the development of a protocol that was, initially, suitable for dispersing pooled mouse adrenals, and which, eventually, after further refinement, could be used for individual adrenal pairs.

Strains DBA/2J, C57BL/Tb and CBA/FaCam were chosen for study, and details, concerning their origins and disease susceptibilities, are described at the beginning of Chapter 2. The strains DBA/2J and C57BL/Tb were selected as they have already been shown to possess contrasting adrenal and gonadal (Shire and Bartke, 1972) functions. Strain CBA/FaCam, apart from intrinsic interest deriving from its reduced testis size (Hayward and Shire, 1974) was thought to be a suitable 'control' strain, having an adrenal physiology that appeared to be intermediate to that of DBA/2J and that of C57BL/Tb, from previous, direct (Badr, 1970), and indirect (Levine and Treiman, 1964; Chapman, 1968) evidence.

Chapter 4 will describe the definition of the corticosterone productive phenotype studied, and also the responses of cells, derived from pooled adrenals of the three strains and some F_1 hybrids, to ACTH and dibutyryl-adenosine-3',5'-monophosphate (dbcAMP).

Cells derived from single adrenal pairs are examined in Chapter 5. Having described the phenotypic distributions of corticosterone productive capacity for genetically uniform stocks, information will be provided concerning segregant backcrosses, as well as any influences exerted by factors linked to two enzymic, and three coat colour, genetic marker genes. Evidence of maternal influence(s) and their interaction with genotype will also be

examined in Chapter 5, as some previous work has suggested their existence, with respect to plasma stress levels of corticosterone (Treiman et al., 1970).

The dry weights, of lymphoreticular target tissue, and testes, will be analysed in Chapter 6, with the main aim of providing evidence of co-adaptation of these organs to the strain-specific adrenal corticosterone productive potentials, described in earlier chapters. Having examined the inheritance of organ growth, in its own right, correlations between organ weights and the defined adrenal phenotype will be used to reveal any marker-linked co-adaptation. Apart from examples of target organ differences in sensitivity already referred to, and others that will be discussed, in greater detail, in the introductory section of Chapter 6, there are two further cases that are worthy of mention here. Dexamethasone has been found to exert a differential effect on mesenteric lymph node cells of CBA/FaCam compared to DBA/2J and C57BL/Tb mice (Taylor, 1976). The implied lack of sensitivity of BALB/Alb spleens to growth-limiting influences, compared with those of C57BL/10A1b, (Mos, 1976), may not be unrelated to the apparent insensitivity of the pituitaries of BALB/c mice, to feed-back inhibition by dexamethasone (Hawkins et al., 1975; Sakellaris et al., 1976). Consideration along these lines may enable conclusions concerning the possible site of action of the ald-2 locus to be reached in the concluding chapter.

CHAPTER 2

MATERIALS AND METHODS2.1 ANIMALS2.1.1 Strains usedDBA/2J

This strain was bred in 1930, from the original DBA stock founded by Little in 1909. It has light brown non-agouti (dba/dba) coat colour and carries the ald-2 gene (Doering *et al.*, 1973) that is phenotypically identical to the androgen-dependent adrenal lipid depletion gene (ald-1) found in AKR mice. This strain, although characterised by unusually large testes (Shire and Bartke, 1972) and marked development of certain androgen-dependent characteristics, concerned with male copulation, does not, however, breed vigorously (Nagasawa *et al.*, 1973), probably as a result of females being poor mothers, both before and after birth, to their offspring. The life-span is relatively long and the strain characteristic pathologies include many neoplastic disorders, particularly mammary adenocarcinomata and lymphomata (Festing, 1979). The mice used for the present work were obtained from Bristol University where they had spent a single generation after importation from the Jackson Laboratory in the USA.

CBA/FaCam

A cross between a Bagg albino female and a DBA male formed the basis for this strain created by Strong in 1920. It was subsequently selected for low mammary tumour incidence (compared to a high selection line from the same original cross; C3H), although current lines have

still got high incidences of this type of cancer (33%) compared with strains such as C57BL (1%) (Smith et al., 1973).

The coat colour is dark black agouti (DBA/DBA): the complete converse of the DBA complement, CBA mice have been shown to possess small testes which, unlike C57BL/10ScSn, are the result of a Y-chromosome effect rather than androgen deficiency (Hayward and Shire, 1974). The strain which is of short to intermediate life-span (Festing, 1979), is of interest, immunologically, as a sex-linked defect has been observed in the responsiveness of B cells to type 3 pneumococcal polysaccharide in the CBA/CaN subline (Scher et al., 1976). The main pathologies are neoplastic; this strain possessing the highest gross tumour incidence of the three strains used in the present work (Storer, 1966), for which breeding stock were obtained from Cambridge University Genetics Department, from a line that had originated from Falconer at Edinburgh University Genetics Department.

C57BL/Tb

This strain, which possesses similar coat colour genes to CBA, apart from being non-agouti (a/a), was founded by Little in 1921, from a mating of Miss Abby Lathrop's stock that also produced other C57 lines. The pre-eminent characteristic of this strain is its apparent lack of dependence on androgens; this being displayed in low plasma levels (Bartke, 1974), testis size (Shire and Bartke, 1972) and male sexual capacity (McGill and Manning, 1976), although the C57BL/Tb subline has relatively normal testis weight possibly due to its ancestry deriving from the main C57Bl line before it was split into the C57BL/6 and C57BL/10 sublines.

Conversely to DBA's, females of this strain are above average dams, probably as a result of being more oestrogen-, rather than androgen-, sensitive. Reproductive performance is therefore good.

Of the three strains studied here, C57BL has the lowest gross tumour incidence (Storer, 1966), and life-span is, generally, long. This strain has been presented as a model displaying the alterations in immune function, specifically the ratio of immunoglobulin M:immunoglobulin G, that accompany autoimmune disorders associated with senescence (Pantelouris, 1974).

The founders of the colony employed in the present research were obtained from Glasgow University Zoology Department whose C57BL/Tb subline was derived from the C57BL/Gr line established by Grüneberg in 1932.

2.1.2 Abbreviations for genetically defined stocks

<u>Stock</u>	<u>Genetic Description</u>			<u>Abbreviation</u>
Parental	{	DBA/2J		DBA
		C57BL/Tb		C57
		CBA/FaCam		CBA
F ₁ Hybrid	{	<u>DAM</u>		<u>SIRE</u>
		DBA/2J	x	C57BL/Tb
		C57BL/Tb	x	DBA/2J
		CBA/FaCam	x	DBA/2J
Backcross	{	(C57BL/Tb x DBA/2J)F ₁	x DBA/2J	ND
		(C57BL/Tb x DBA/2J)F ₁	x C57BL/Tb	NC
		(DBA/2J x C57BL/Tb)F ₁	x DBA/2J	OD
		(DBA/2J x C57BL/Tb)F ₁	x C57BL/Tb	OC
		C57BL/Tb	x (C57BL/Tb x DBA/2J)F ₁	CN
		(CBA/FaCam x DBA/2J)F ₁	x DBA/2J	QD
		(CBA/FaCam x DBA/2J)F ₁	x CBA/FaCam	QB

2.1.3 Husbandry

Mice were caged in standard mouse cages (Cambridge Model: Assoc. Crates) with Oakite 613 mineral bedding.

They were kept at $22^{\circ}\text{C} \pm 1^{\circ}\text{C}$, experiencing a twelve hours light: twelve hours dark cycle (lights on at 6.00 a.m.). Mouse food (Oxoid breeding diet) and tapwater, contained in water feed bottles, were freely available to all animals. Humidity was not controlled.

2.1.3.1 Breeding of pure strains

Six to eight week old sibs were mated in pairs and occasionally in threes (one male, two females) depending on available cage space. A little shredded paper was included in mating cages to provide a nest for the young.

Matings, which usually gave plentiful good-sized litters, were subject to slight decline in fecundity during the winter months.

Unfortunately, not all genotypes displayed equally good breeding characteristics: DBA females tending to devour their new-born, C57 males being reticent copulators, as mentioned already in Section 2.1.1. The hybrid F_1 (DBA female x C57 male) was thus the most difficult stock to obtain; both parents being inefficient at breeding.

Young mice were weaned three weeks after birth and were caged, up to six to a cage, having first been separated according to sex.

Many matings were fruitful for over a year, but new matings were usually established every three months. Hybrid F_1 and backcross matings were initiated and maintained in an identical way except older parents were sometimes used according to the availability of stocks.

2.1.3.2 Caging prior to experimentation

An awareness of potentially large genotype x environment interactions with adrenal function ensured that animals were exposed to the minimum of obvious and avoidable stress.

It has been shown that fighting (Lemondé, 1959), particularly submissiveness within large groups (Davis and Christian, 1957), increases adrenal activity.

Any cage of mice exhibiting evidence of fighting was not used for experimental purposes.

The ideal animal housing, from the experimental point of view, would have been two to a cage from weaning. This would have been optimal on the long term in that this constant number would have been unlikely to constitute a stressful novelty such as isolation or overcrowding (Dechambre and Gosse, 1973). This small number would also have been ideal in the short term, during experiments, as it would greatly reduce the risk of stressing animals as panic-stricken cagemates were removed, particularly since there is some evidence that adrenal cortical activity may be under direct neuronal, as well as hormonal, control (Ventura et al., 1976; Engeland and Dallman, 1976).

As caging facilities were unable to meet this ideal arrangement for any length of time, a compromise was sought whereby littermates were stored upto six to a cage until ten days before experimentation. At this time, having checked the number, sex and fitness of animals, cagefuls of six were split into two new cagefuls of three. Cages of five were split into threes and pairs, although the latter were avoided where possible as they were felt to represent a novelty approaching that of complete isolation. Cages of four were thus left undivided.

Such recagings, however, where possible, were avoided by preferential utilisation of mice, stored two to four animals to a cage.

It is unlikely that handling, per se, produced significant adrenal changes as no hypertrophy was observed in descendants of wild house mice when they were handled twice daily for ten days (Davis and Christian, 1957). They may have admittedly possessed genetically unreactive adrenals, but as the same work revealed a marked hypertrophy when animals were overcrowded, this seems improbable.

The storage of mice, upto six to a cage, from weaning was also considered to be unlikely to produce adrenal activities significantly different from paired mice, as only extreme crowding, in rats, was found to elevate mitochondrial 11-beta-hydroxylation; moderate crowding causing no such effect (McCarthy *et al.*, 1976).

At the time of dissection, maimed, diseased or stressed (as judged by abnormally enlarged adrenals) animals were not used for experimentation.

2.2 STEROID EXTRACTION AND ANALYSIS

2.2.1 Introduction

A few initial experiments, in which methylene chloride extracts were chromatographically purified before fluorometric analysis, indicated that when maximally stimulated with ACTH, incubates contained high proportions of corticosterone. This was corroborated by the demonstration of high proportions of authentic corticosterone in unchromatographed samples.

In order to study as many single adrenal pairs as possible, steroid analysis had to be simple. The bulk of experiments, therefore,

did not include thin layer chromatography but consisted of the assay of crude methylene chloride extracts. The slight loss in phenotypic clarity, resultant from this, was, however, likely to be more than compensated by the greatly increased resolution of segregant classes by the study of more individual mice.

2.2.2 Glassware preparation

Glassware used, not only for extraction, but for all other experimental stages, was cleaned and siliconised in the following fashion.

An initial wash in ordinary laboratory detergent was carried out for non-radioactive items; this wash being in Decon for radioactive ones.

After thorough rinsing, glassware was then completely immersed in concentrated nitric acid, for at least 24 hours. This was followed by thorough rinsing and oven drying.

Siliconisation was carried out using "Repelcote" (Hopkins and Williams) siliconising fluid. This was only semi-permanent and had to be repeated after each acid-wash. The process was carried out at room temperature; ensuring that the entire inner surfaces of articles had been in contact with the solution.

Excess carbon tetrachloride was allowed to evaporate from the glassware by leaving to drain in a fume cupboard. Excess hydrochloric acid, formed in the siliconising reaction, was then removed by rinsing in warm water and, finally, in glass-distilled water.

All pipettes used in extraction were double-washed with methylene chloride.

2.2.3 Corticosterone standard storage

Bulk dilutions of corticosterone (corticosterone alcohol, Sigma Chemicals) were made up using ethanol (specially purified for the determination of 17 keto-steroids, BDH) as solvent, in 50 ml flasks. (All glassware was acid-washed and siliconised.) The solutions, which were usually 250 nanograms/ml and 500 nanograms/ml, were then dispensed, in 1 ml aliquots, into small storage tubes (7 x 50 mm), which were then sealed with wax film and stored at -20°C . Bulk storage was avoided to prevent accumulation of contaminants that could be potentially introduced during withdrawals of aliquots, for each extraction.

2.2.4 Steroid extraction from steroidogenic incubates

Incubates, from which steroid was extracted, were the end-products of the adrenal dispersion and ACTH (dbcAMP) incubation methods which will be described in Chapter 3.

The extraction method was that used for extracting corticosterone and cortisol from human blood (Butte and Noble, 1969).

Each extraction was carried out with upto 26 incubates (stored at -20°C after termination of the steroidogenic incubation), 4 corticosterone standards, and 2 distilled water blanks.

Stoppered tubes (Quickfit, 15 x 100 mm) were used for the actual partitioning process, while one set of test tubes received extract to be analysed fluorometrically, another set receiving samples that were stored, hermetically, at -20°C , and later used in the demonstration of authentic corticosterone content (Section 2.2.6).

Corticosterone standards (250 nanograms and 500 nanograms in ethanol) were removed from storage at -20°C and transferred to 4 stoppered tubes. Two standards at each concentration were used. The

ethanol was then force evaporated by placing tubes in a water-bath (45°C) and passing oxygen-free nitrogen over the solvent surface, via Pasteur pipettes clamped about 4 cms above it.

When only steroid remained, 1 ml of distilled water was added to the stoppered tubes.

Having defrosted the incubates, 0.2 ml distilled water was added to each, to give a total volume of 1 ml. 5.5 mls of methylene chloride (Koch Light, Puriss. grade) were added to the water blanks and standards. Only 5 mls of solvent were placed in the other stoppered tubes as 0.5 ml was used to wash the incubation vials after their contents had been transferred to the solvent tubes, with the minimum of turbulence. All tubes thus, eventually, contained 5.5 mls methylene chloride with 1 ml incubate, or water.

Half the total number of tubes (up to 16) were stoppered and placed in a small rack so that one duplicate from each incubate, standard and blank were inverted, together, exactly 70 times. Having repeated this for the remaining duplicates, all tubes were then centrifuged at 0°C at 1500 g for 10 minutes.

The aqueous phase was then removed from each tube and 5 mls of extract were removed, 2 mls of which were placed in tubes for fluorometric analysis, while 3 mls were placed in storage tubes. The 5 ml pipette used for this, in addition to ordinary laboratory washing, had been washed, twice, with methylene chloride. Water blanks were transferred first and the pipette was washed with chloroform between each pair of duplicates.

Both sets of tubes were allowed to remain at 4°C for 24 - 36 hours, after which time some evaporation had taken place. All extracts

were then evaporated under oxygen-free nitrogen in the manner described earlier, and fluorometric assay was carried out on the 2 ml extracts, while 0.5 ml ethanol was added to the storage tubes, before placing them at -20°C .

2.2.5 Thin layer chromatography

Silica gel (F_{254} , Merck) plates (5 x 20 cm) were hand-poured, using a pneumatic device that aligned the upper surfaces of the glass plates, to give a layer of about 0.3 mm thickness. Before use, plates were acid-washed (having been Decon-washed, if radioactive) and given a final polish with ethyl acetate, just before pouring, to remove any contaminants collected during handling.

The 3 ml storage extracts, referred to in the extraction procedure, consisting of samples, standard and blanks, were evaporated under nitrogen as before, and 0.05 - 0.1 ml of ethanol were used to dissolve residues, by gentle vortexing.

A chromatography standard corticosterone (corticosterone alcohol, Sigma Chemicals) solution was used at a concentration of about 10 mgms/ml ethanol.

Plates were activated at 120°C , for one hour, and pre-run in the chloroform-ethanol solvent used for chromatography. Margins were drawn on both sides of plates to reduce edge effects, and plates were divided into two or three lanes, depending on the number of chromatography standards accompanying the extract. (Two chromatography standards, flanking an inner sample lane, were used to increase the accuracy of corticosterone location.)

Samples were applied carefully, to avoid damaging the silica gel surface, and also to keep the origin spot as small as possible.

Plates were then run in a Shandon chromatography tank (designed for 5 x 20 cm plates) in a saturated atmosphere of the solvent combination used; 95 parts chloroform (BDH, spectroscopic grade) with 5 parts ethanol.

After a running period of about 45 minutes, plates were removed and allowed to dry.

Chromatography standards were visualised by applying a weak solution of iodine in diethyl ether to the expected regions of the standard lanes. Subliming iodine and ether vapour were prevented from interacting with steroids in the incubate sample lane by placing a hair drier near the plate during the visualisation procedure in such a way as to create an air current away from lanes not being visualised.

A band of silica gel, roughly 25 mm wide, was then scraped from the extract lane, in a region parallel to the visualised standard(s), using a razor blade, mounted on a spatula.

Steroid adsorbed to this silica gel was then eluted by four separate washings with 1 ml of 1:1 ethyl acetate:methanol (BDH, analar grades) so that 4 mls of eluate were eventually transferred to one tube. The eluates were then stored for 24 - 36 hours at -20°C until analysed fluorometrically.

Despite attempts to rigorously control the procedure, recovery of corticosterone in incubate and extraction standard eluates was variable. Tritiated corticosterone (Radiochemical Centre, Amersham) was used to estimate recovery in some experiments and was added before methylene chloride extraction. Scintillation counts of samples, in

the scintillant, NE 250 (Nuclear Enterprises), from chromatography eluates enabled corticosterone recovery to be estimated.

2.2.6 Authentication of corticosterone in unchromatographed methylene chloride extracts

Although the major steroid in extracts from cell incubates appeared to have identical fluorometric characteristics to corticosterone, it was crucial to establish that it was, irrefutably, corticosterone. This was done by thin-layer chromatographic comparison with authentic steroids, before and after acetate derivative formation, in three different solvent mixtures:

1 volume ethyl acetate	:	1 volume isohexane
95 volumes chloroform	:	5 volumes ethanol
115 volumes benzene	:	35 volumes acetone

Three samples were compared; extract of cell incubates, corticosterone extraction standards, and freshly made up solutions of authentic steroid (corticosterone alcohol or corticosterone acetate, Sigma Chemicals).

The first two samples were pools of extracts from many experiments, collected over two years, that had been stored under ethanol at -20°C .

Acetylation, by a standard method (Zaffaroni and Burton, 1951), was carried out by permitting the reaction to occur overnight, at room temperature, in 3 volumes acetic anhydride:5 volumes pyridine. (All solvents were of at least analar grade, ethanol, chloroform and methylene chloride were aristar grade, BDH).

Samples were applied and run, in three lanes on the same plate, in the fashion described in Section 2.2.5, and steroids were visualised

by means of iodine vapour. In every case, the major steroid component (dark brown spot) was of identical R_F for incubate sample, extraction standard and fresh standard. Other steroids were present in considerably smaller amounts as evidenced by very faint and small bands.

It was thus concluded that not only was the major steroid in incubation extracts corticosterone but that it appeared to comprise a very large part of the extracts.

This preponderance of corticosterone, in the incubation media of cells maximally stimulated by steroidogenic agonist, is consistent with findings in the present work, and others using isolated adrenal cells (Sayers et al., 1971; Barofsky et al., 1973), that the levels of corticosterone in maximally stimulated cell incubates increase dramatically (about 10 - 20 times the basal level) when steroidogenesis is maximal. Differences between corticosterone concentration estimates from crude and chromatographically purified samples will be shown in Section 4.2.1, to be so small, under maximal conditions, that the chromatographic purification step became unnecessary.

2.2.7 Fluorometric assay of corticosterone

A number of variants exist on the original technique of Sweat (1954) for fluorometrically determining nanogram quantities of steroids such as corticosterone and hydrocortisone. Meaningful and time-saving analysis of unpurified steroid extracts has been sought (Silber et al., 1958) as well as greater sensitivity (Glick et al., 1963).

The basic principle: the property of certain types of steroid molecule to exhibit fluorescence in an extremely acidic solution, has remained the corner-stone.

The first of the following sub-sections describes the characterisation of the assay used for the present work, particularly the essential Beer's law requirement for linearity of relationship between fluorophor concentration and fluorescence.

The other sub-section describes the technique used to assay corticosterone in experimental extracts.

2.2.7.1 Characterisation of assay

Before use, experimentally, the assay, described in 2.2.7.2, had to be shown to be the resultant of a simple linear relationship between steroid concentration and fluorescence, observed in acidic ethanol solution.

Table 2.1 gives the relative fluorescences (%'s) of different standards of corticosterone observed on several occasions. The fluorimeter sensitivity and gain setting were different with each comparison and %'s were thus not directly comparable in the absence of a further standard, such as quinine sulphate. Linearity was discernible within experiments, but an idea of how much it existed for all data was possible by correcting according to mean % fluorescence of one nanogram, for each experiment. Values corrected to the specific fluorescence of the final experiment, are given in the final column of Table 2.1.

Means, formed from the corrected % fluorescences, were found for each standard value, and are given in Table 2.2.

Table 2.1. The relationship between corticosterone concentration and its fluorescence in acidic ethanol.

Experiment	Standard (ngms)	% fluorescence	% fluorescence x $\frac{0.985}{\text{S.F.}}$
1 (duplicate values) S.F. = 1.081	500	545.3	496.9
	50	54.3	49.5
	5	5.33	4.9
	0.5	0.83	0.76
2 (duplicate values) S.F. = 1.807	32	57	31.1
	24	40.75	22.2
	12	26.25	14.3
	4	6.25	3.4
3 S.F. = 0.624	200	130.65	206.1
	100	60.65	95.7
	50	30.65	48.4
4 S.F. = 0.898	100	89.75	98.5
	50	45.25	49.7
	25	22.25	24.4
5 S.F. = 1.062	500	531	492.5
	50	53.1	49.3
6 S.F. = 0.919	400	372.5	399.4
	100	87.5	93.8
	50	47.5	50.9
7 S.F. = 0.985	400	392	392
	200	204	204
	100	96	96
	50	49	49

S.F. = Specific Fluorescence = % fluorescence/concentration.

Table 2.2. Mean fluorescences for different corticosterone concentrations

Standard (ngms)	N	mean %	Standard (ngms)	N	mean %
0.5	2	0.76	32	2	31.1
4	2	3.4	50	8	49.5
5	2	4.9	100	5	96.5
12	2	14.3	200	2	205.1
24	2	22.2	400	2	395.7
25	2	24.4	500	3	495.4

N = number of individual observations.

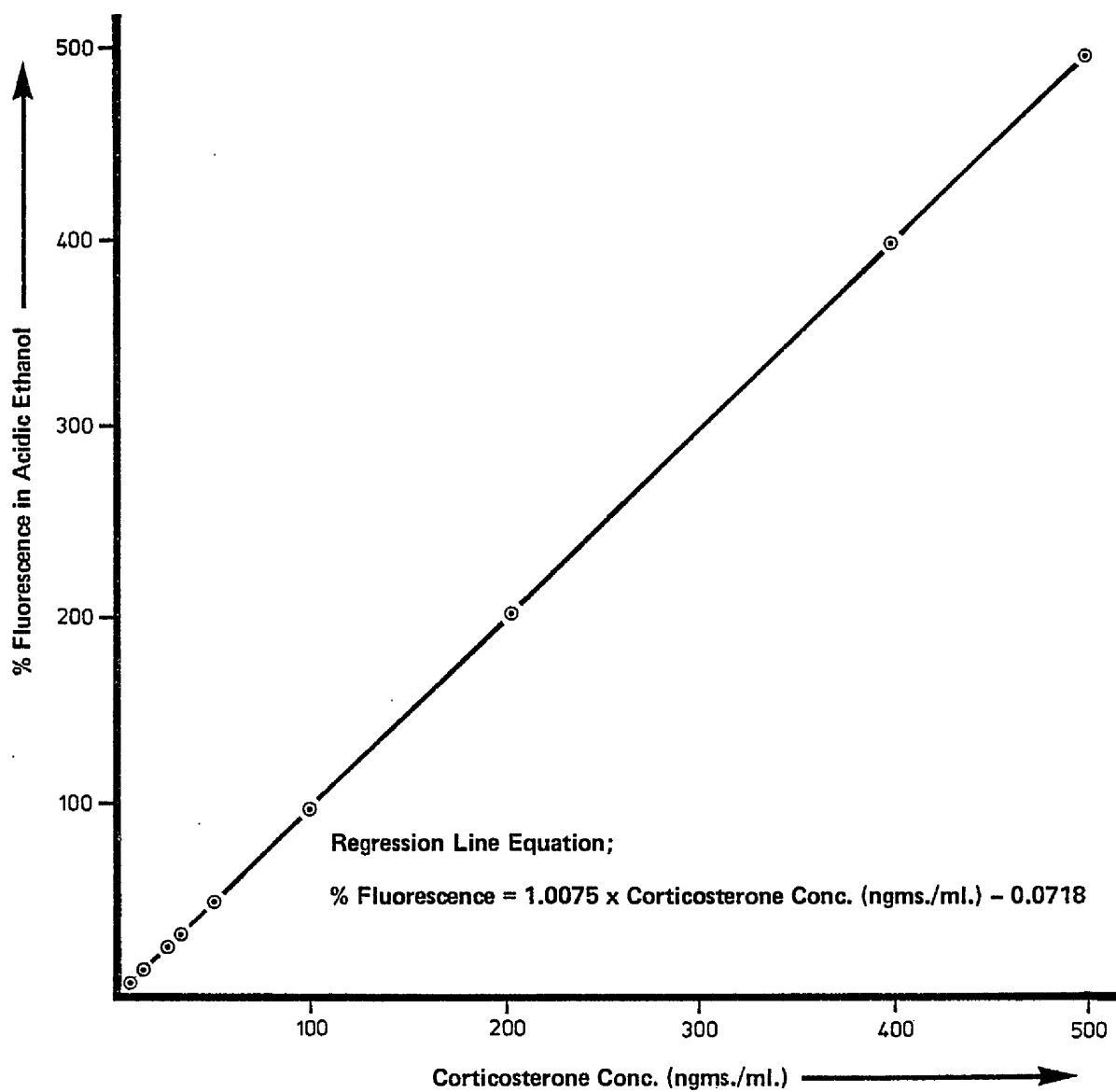
The regression line formed from these plotted values (Figure 2.1) indicated a high degree of linearity. A possible breakdown in this relationship may have been demonstrated at steroid concentrations of between 500 and 4,000 picograms and this may have represented a limit to the assay sensitivity. This limit was only between 0.5 and 4% of the lowest measurements made on experimental incubates.

Scans carried out on these corticosterone standards showed that fluorescence was maximal when the excitation wavelength was 470 nm and the emission wavelength was 523 nm.

2.2.7.2 Assay procedure

The sulphuric acid:ethanol reagent used to examine extracts, fluorometrically, was similar to that described by Butte and Noble (1969) consisting of 9 parts acid to 5 parts ethanol. It was usually made up,

Figure 2.1 Linear relationship between corticosterone concentration and fluorescence in acidic ethanol.



200 mls at a time, and stored at 4°C for a maximum of 14 weeks. Sulphuric acid (BDH, aristar grade) was added very slowly to pre-chilled ethanol, and heat produced by each addition was rapidly dissipated by thorough agitation of the stock bottle in a bucket of ice chips.

Pasteur pipettes, required to transfer samples to the 1 ml silica cell used in the Baird Atomic (Model fluoripoint FT1010) fluorimeter, as well as the 10 ml pipette used to add acid:ethanol to sample tubes, were given two washes with chloroform before use. All pipettes were also plugged with cotton wool to avoid fragments of highly fluorogenic rubber, from pipette fillers and teats, falling into samples.

The usual protocol that was followed before and during analysis is now described.

Methylene chloride extracts (2 mls), that had been stored at 4°C since extraction, were force-evaporated using oxygen-free nitrogen and replaced at 4°C.

In order for fluorescence to be observed between 45 and 60 minutes after addition of acid:ethanol, additions of reagent occurred at 15 minute intervals to groups of 10 samples. Within each group consisting of one from 10 duplicates, the order of analysis was alternately reversed so that if one duplicate was read at the beginning of a 15 minute reading period, the other duplicate, in the next group, was read at the end. This meant that, assuming any slight increase in fluorescence during this period was roughly linear, the means of all duplicates represented fluorescences that were read 52.5 minutes after addition of reagent. Any single incubate was thus read as near this time as possible.

Fluorescence was developed at room temperature after adding 1 ml of acid:ethanol to 10 tubes in a group and vortexing for 20 seconds.

The fluorimeter was switched on 45 minutes before use to allow stabilisation of the light source and also of the photo-amplification electronics. About 5 minutes before readings were taken the electronics were zeroed in the absence of light. The cuvette (which had been thoroughly rinsed of the concentrated nitric acid in which it was kept) was then rinsed with some acid:ethanol and then, avoiding contamination with finger marks by holding at the corners, it was filled with the first sample, which was always the water blank extract. Having set the excitation wavelength at 470 nm and emission at 523 nm, the sensitivity of the machine was maximised by adjusting the light path, with the first sample interrupting it. The only control altered after this juncture was the coarse gain (fine gain was not used) according to the differences in fluorescence of different samples.

Fluorescences were read as %s, to the nearest 0.5%, and the cuvette, which always presented the same face to the light source (identifiable by a manufacturer's marking) was rinsed with a little acid:ethanol between samples.

2.3 CELL COUNTS USING COULTER COUNTER

2.3.1 Introduction

The production of adrenal cell suspensions and their incubation is described in Chapter 3. Dilutions from such suspensions were originally counted, using a haemocytometer; this technique gave good estimates but their accuracy was dependent on the number of samples of cells counted. This method was found to be impracticable, without losing a significant degree of accuracy, when more than a few suspensions were being compared.

By the time many individual adrenal pairs were dispersed within one experiment, it had become essential to use a faster counting method that would enable final resuspension volumes of different cell suspensions to be calculated quickly during the final cell centrifugation. A regularly used and well maintained Coulter counter (Coulter Electronics, model D) was found to combine speed with the necessary accuracy.

A description of haemocytometer counts and the transition to Coulter counting by the selection of an appropriate threshold setting is described in the first of the following sub-sections.

About half-way through the total experimental period, the Coulter counter became defective and was replaced. The new counter was used at the same threshold setting as the old, until it became apparent that it was under-counting by, approximately, a factor of 2.5. Two factors disguised this fact for some time; moderate variation in cell yield between experiments and, more importantly, the uncontrolled inhibition of ACTH responsiveness by a factor (described in Chapter 3) that balanced the lowered cell counts, giving apparently normal steroid productions per viable cell. The second sub-section describes evidence for this Coulter counter difference.

The final section indicates the altered threshold adopted and the method of correcting counts performed at the wrong threshold.

2.3.2 Haemocytometer counts, and Coulter counts using an appropriate threshold setting.

The correct chamber volume in the haemocytometer was ensured by pressing the special coverslip onto the glass surrounding the counting chambers so that interference patterns were observed. Drops of suspension were introduced into the chambers by capillarity and cells were allowed

to settle onto the etched glass grid for about a minute.

Cell suspensions consisted almost entirely of single cells, although small clumps of 2 - 6 cells were occasionally found. Clumps larger than this were very rare and were not included in the count.

Both cortical and medullary adrenal cells were counted; the small numbers of blood and fat cells being ignored. This relatively time-consuming technique was replaced by the use of a Coulter counter; a change that was essential if large numbers of adrenal suspensions were to be studied simultaneously.

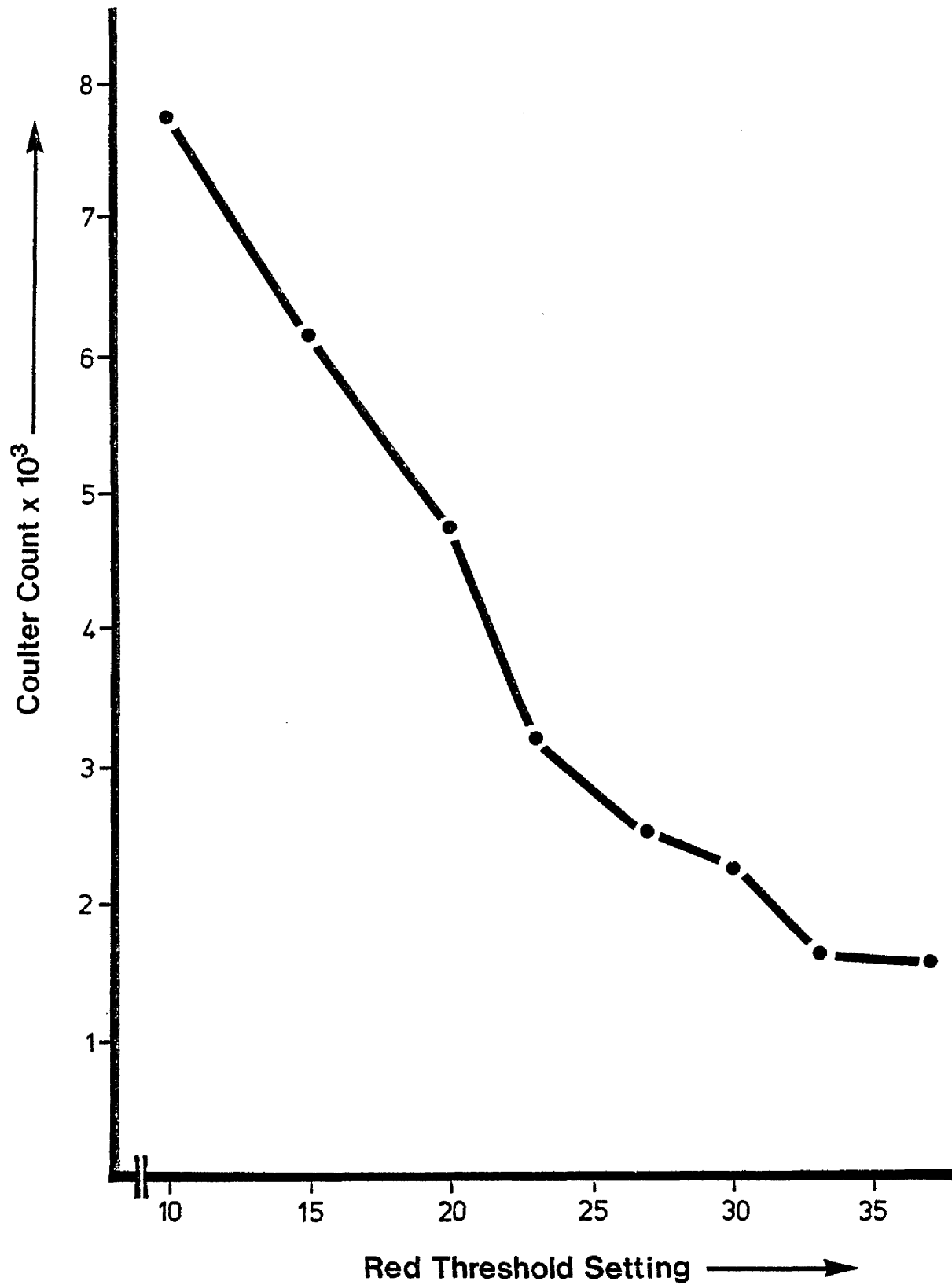
The transition from one method to another required a suitable threshold setting, on the Coulter counter, to be selected, that would give cell counts similar to those estimated using the counting chamber.

The first experiment combining both methods, using adrenals from 7 CBA females, compared Coulter counts achieved at different red blood cell threshold (T) settings with a haemocytometer count. Dilutions of suspension (1 in 100 - 200) were made in filtered, sterile saline (stored at 4°C) and 0.5 ml samples were counted by Coulter counter. Between 2 and 4 samples were counted at eight threshold settings (between 10 and 37 in the red counting mode). The aperture current was always at the same setting for all Coulter counts carried out in all experiments.

A count versus T (threshold setting) relationship for this experiment is depicted in Figure 2.2, from which a T value between 20 and 23 was thought to give a count equivalent to that obtained by haemocytometer. Interpolation of this graph indicated that $T = 21$ might give exact correspondence.

A following experiment, using a suspension of 5 male DBA adrenals, indicated that counts by the two methods were not significantly different,

Figure 2.2 CBA female adrenal cell Coulter count versus red threshold setting.



as judged by t test, when the Coulter threshold setting was $T = 21$. This setting thus became the one used in all future experiments comparing genetically defined adrenals, although a number of comparisons continued to be made in subsequent experiments to obtain more exact evidence of the degree of correspondence, as shown in Table 2.3.

Haemocytometer samples in Table 2.3 consisted of the number of cells in 16 small squares; many samples were thus drawn from one grid. Coulter counter samples consisted of the cells counted within 0.5 ml aliquots of a saline dilution.

From these comparisons it can be seen that when many dispersions are considered, the Coulter counts were significantly lower than haemocytometer counts by about 16%.

As it is probable that this percentage, of the entire adrenal cell population not counted, largely consisted of the smaller medullary cells, an inadvertent reduction in the contribution of non-steroidogenic cells in the assessment of corticosterone produced per cell, in response to ACTH must have occurred. The disadvantage of thus being unable to detect any medullary/cortical interactions may have been greatly outweighed, however, by a reduction in a probably inert but genetically variable (Shire and Spickett, 1968a) component of the cell count.

Attempts to distinguish possible cell types as plateaux in the threshold versus Coulter count graphs were largely unsuccessful as might be expected with suspensions containing many cell types that were not entirely clump-free. The combined data of two experiments (in which counts were very similar), shown in Figure 2.3, however, indicates the presence of probably one, and possibly a second, cell type whose plateaux begin at the vertical dotted lines.

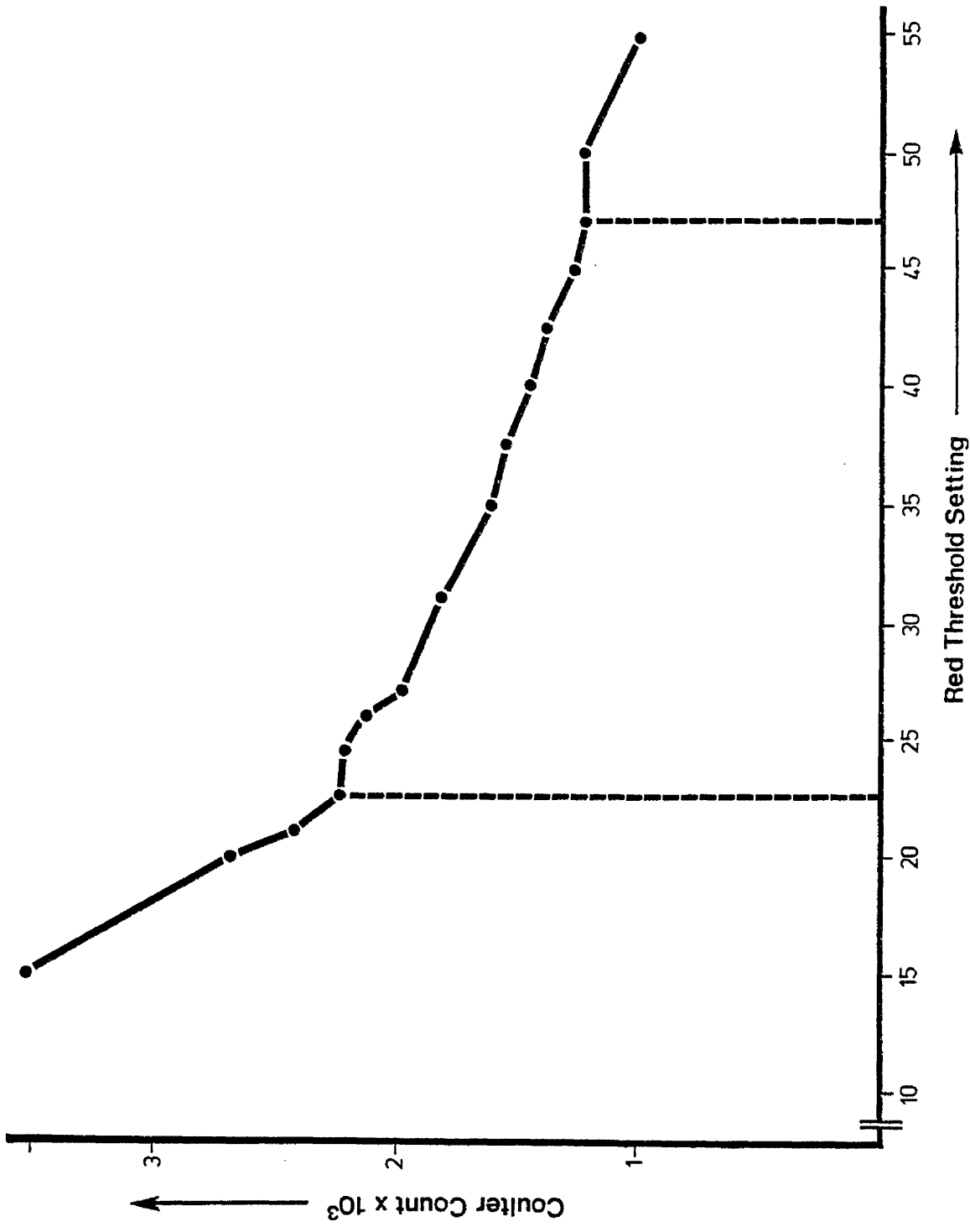


Figure 2.3 Combined C57 male adrenal Coulter counts versus red threshold setting.

Table 2.3. Comparison of haemocytometer and Coulter counts. (Coulter and haemocytometer counts represent the cells/ml x 10⁵ original suspension.)

Expt.	Haemocytometer		Coulter Counter		t Tests Probabilities(%)	Ratio mean Coulter to mean haemocytometer		
	Mean	S.D.	Sample Size	Mean S.D.			Sample Size	
10	7.109	1.799	32	7.425	0.4143	4	NS	1.044
14	8.864	1.928	20	6.819	0.1514	6	0.1	0.769
14	6.457	1.559	23	5.065	0.0831	6	0.1	0.784
18	7.129	1.498	7	6.682	0.0849	6	NS	0.937
18	7.561	1.558	6	6.403	0.0967	5	NS	0.847
19	7.850	1.528	10	6.286	0.1660	6	1.0	0.801
19	8.650	2.593	10	7.226	0.0838	5	NS	0.835
20	5.841	2.020	5	4.452	0.0258	3	NS	0.762
20	6.250	1.792	4	5.048	0.0202	2	NS	0.808
21	7.833	1.232	9	6.840	0.0520	3	5.0	0.868
21	8.160	1.909	9	6.139	0.1461	3	2.0	0.752
MEAN: 0.835								

2.3.3 Evidence suggesting different counting characteristics of a replacement Coulter counter

As mentioned earlier, the altered counting characteristics of the replacement Coulter counter only became recognised when the disguising effect of a factor inhibiting steroidogenesis had been removed. Comparisons were then made between count versus threshold graphs constructed before, and after, machine replacement. At the time, the differences in slope characteristics were regarded as strong evidence of differing counting performances, so haemocytometer counts were not carried out. Later, when the manufacturers of the machines (Coulter Electronics) were consulted, they advised that differences between such count versus threshold graphs, although indicative that counts were probably not made under the same conditions, were of little quantitative value.

Normal counts were obtained when the replacement machine threshold setting was about 2 - 3, but indirect evidence was sought to substantiate the possibility that the replacement Coulter counter was undercounting, with respect to the first, by a factor of about a half, when threshold = 21 was used.

Such evidence concerning total cell yield, total steroid output, and response to dibutyryl cyclic adenosine monophosphate (dbcAMP), indicated the strong possibility of such a difference, between the two machines, existing.

Total cell yields, representing the mean of 12 individual dispersions for each experiment, were formed from counts at $T = 21$ on each counter. A number of experiments, before and after replacement of the first Coulter counter, were compared. The proportions of

different strains of animal comprising each group were roughly similar so that any difference, especially a gross one, would have been most unlikely to be due to genetic factors affecting either adrenal size or dispersability. Dispersions carried out after arrival of the second machine all used the same collagenase incubation volume; 0.25 ml, whereas some of the earlier experiments used the larger volumes, indicated in Table 2.4.

A small amount of evidence, presented in the next chapter, suggested that doubling the collagenase volume within the same experiment produced higher cell yield probably due to mechanical effects. It will also be made clear how increasing the collagenase incubation rate of agitation produced the same effect. As such increases in oscillation rate accompanied the decreases in collagenase volume size in Experiments 24 - 35, in Table 2.4, they probably formed a homogeneous group with the remaining experiments (36 - 41) using the smallest incubation volume (0.25 ml). The t values for comparisons between the 0.25 ml group (experiments 36 - 41), and all other incubation volume groups (experiments 24 - 35) (separately and together) were all less than unity even when the standard deviation of the larger group was used to form the standard deviation of the difference between means, on the assumption that it was a more accurate estimate of group standard deviation.

Having thus considered the 'before' and 'after' groups as similar and comparable, a t test between the group means gave a probability of less than 0.001.

The ratio of 'before' cell yield to 'after' was found to be 2.33; roughly the factor by which steroidogenesis/cell, observed after

Table 2.4. Comparison of total cell yields, computed from cell counts performed at threshold setting = 21, for the original and the replacement Coulter counters.

Expt.	Original Coulter counter		Replacement Coulter counter	
	Collagenase vol.	Total cells x 10 ⁵	Expt.	Total cells x 10 ⁵
24	0.5	3.9096	42	1.6352
25	0.6	5.0751	46	1.7368
26	0.7	2.1804	47	1.3234
27	0.4	3.7352	48	1.3598
28	0.4	3.3320	49	1.6614
29	0.4	3.7020	50	1.8980
30	0.35	4.8832	54	1.5150
31	0.35	3.4752	55	2.0192
33	0.35	3.9072	56	1.4396
34	0.35	3.3600	57	1.6340
35	0.4	2.8896	58	1.3971
36	0.25	3.0408	59	1.5233
37	0.25	2.8618	60	1.6396
38	0.25	3.1064	61	1.4028
41	0.25	4.5630	63	1.4889
			64	1.3361
			65	1.2865
<hr/>				
	MEAN:	3.6014	MEAN:	1.5469
	S.D.:	0.78989	S.D.:	0.20560
	N :	15	N :	17

t = 9.79 p less than 0.001 (two-tailed test between overall mean counts obtained for the two different Coulter counters)

Coulter replacement, using pure bovine serum albumin (BSA), exceeded uninhibited values obtained, using the first Coulter counter.

As stated earlier, it was only when BSA purity was controlled that this approximate doubling of apparent steroidogenesis/cell became obvious and suggested that the second Coulter counter might be undercounting at $T = 21$.

An alternative explanation, that use of an exceptionally pure BSA allowed steroidogenesis to be less inhibited than it had hitherto been observed, was not supportable on grounds relating to total steroid production and also response to dibutyryl cyclic adenosine monophosphate (dbcAMP).

Considering the first of these; a direct comparison was made of mean steroid content of incubates before and after BSA was controlled. This consisted of taking all obviously uninhibited values for each strain, observed before use of constantly pure BSA, and matching them with an equal number of randomly chosen values obtained, for the same strain, after BSA control.

Table 2.5 shows the results of this comparison; N being the number of individual values in each group for a particular strain.

Although the number of individuals and strains available for comparison was limited, the data of Table 2.5 indicated that as total steroid output remained virtually the same, the apparent doubling of steroidogenesis per cell must have resulted from the undercounting, by about 100%, by the replacement Coulter, implied by the earlier cell yield comparisons given in Table 2.4. These total steroid comparisons also implied that the BSA batch used during the period of control was little different from those batches in the past that had also shown no

Table 2.5. Comparison of total steroid yields for uninhibited experiments before and after purity of BSA was controlled.

Strain	N	NGMs STEROID/INCUBATE			
		Before BSA control		After BSA control	
		Mean	S.D.	Mean	S.D.
DBA	4	122	27	132	19
C57	9	253	131	257	81
NC	6	309	148	334	162
OD	6	112	37	115	53

inhibitory properties.

Such similarities in total steroid output (Table 2.5) could also have been observed if the cell yield had been truly and dramatically halved and the control BSA possessed some quality (be it greater absence of inhibitors, or presence of some agonist) that doubled these cells' steroidogenic response. This combination of two unlikely events is much less probable than the proposal of a single counting defect.

The improbability of the controlled BSA possessing different qualities from previous BSAs that had lacked inhibitory effects, was augmented by consideration of responses to dbcAMP.

Experiments, using duplicate incubates of pooled DBA cells, maximally stimulated with 7.5 mM dbcAMP or 2.5 mU ACTH/ml, gave the responses shown in Table 2.6 (pgms steroid produced/cell/hour) in controlled and inhibitory BSA, and using the first and second Coulter counters. Similar incubates of N cells were counted with the second

Table 2.6. Responses to ACTH and dbcAMP in experiments using inhibitory and controlled BSAs, and also the original and replacement Coulter counters.

Inhibitory BSA				Controlled BSA			
Strain	Counter	dbcAMP	ACTH	Strain	Counter	dbcAMP	ACTH
DBA	Original	2.824	2.497	DBA	Replacement	5.631	5.181
N	Replacement	9.983		N	Replacement	10.223	

counter but differed in type of BSA. The ACTH response for DBA cells, in controlled BSA, was obtained within the same experiment as the dbcAMP response. In inhibitory BSA, as the ACTH value was meaningless, the mean DBA value for all pooled incubations (see Chapter 4) was used.

The lack of effect of inhibitory BSA on N cell dbcAMP response supports the finding, discussed more fully in Chapter 3, that contaminants in such BSAs appeared to affect, almost exclusively, the ACTH response.

Having eliminated any significant dbcAMP x BSA type effects, the observed difference in the DBA steroid productions/cell in response to dbcAMP could only have resulted from a reduced cell count using the second counter, as total steroid output has already been shown to have remained constant.

The exactly parallel difference observed in ACTH responsiveness suggested the same variable to be the cause.

In summary, two separate phenomena indicated that the second Coulter counter was undercounting, possibly by a factor of about two,

with respect to the first. One comparison, cell yield, indicated such a difference; with no overlap in the distributions, between those estimates formed before and those formed after counter replacement. The observation of apparently doubled steroidogenesis with the use of contaminant-free BSA suggested underestimation of cell count rather than unprecedented conditions (less inhibition of the response than occurred in earlier uninhibited experiments) allowing greater steroidogenesis to occur. This was demonstrated by the lack of any increase in total steroid content per incubate for different strains, and also by the doubling of the dbcAMP response, which was largely independent of BSA inhibition.

2.3.4 Threshold setting alteration or use of a correction factor to render counts from the replacement Coulter counter comparable with those of the original counter

The first of the following sections describes the use of a new threshold setting on the replacement counter that was selected by comparison of the total count v. threshold relations of the two counters. The increased count is related to a possible cause of the counting difference.

The second section deals with the method used for correcting counts made at the original threshold ($T = 21$) in experiments that betrayed no evidence of a cell count difference due to the inhibition of steroidogenesis by BSA contaminants.

2.3.4.1 Selection of a new threshold setting on the replacement Coulter counter

Once the data had accumulated sufficiently to show, in the ways described in Section 2.3.3, that it was virtually certain that a counting difference existed between the two Coulters used, attention

became focussed on differences in total count v. threshold (T) relationships, for both counters. Although comparisons of such a type could only be expected to indicate gross differences, it was noted that the curves for the replacement counter were all of smaller general negative slope than those obtained over the same threshold range, using the original. In order to try and normalise this it was necessary to find a lower threshold (T varies inversely with total count) that could be regarded as equivalent to the original threshold, $T = 21$, on the original machine.

The shape of the count v. threshold curve originally found for the first counter (see Section 2.3.2) possessed an apparent plateau at about $T = 23$. Evidence of an analogous plateau was found at $T = 4.5$ on the replacement. A threshold of $T = 2.5$ on this counter was judged to be equivalent to $T = 21$ on the original counter, both in terms of general count v. threshold slope and shape.

Counts at both $T = 21$ and $T = 2.5$ were made in all experiments, thereafter, and the ratio (mean for all genotypes) of the latter to the former was found to be 2.480.

Such a gross difference was suggested by the manufacturers (Coulter Electronics) to be due to the use of different counting tube aperture diameters. Subsequent enquiry revealed that although the laboratory had possessed two counting tubes: 140 μ and 100 μ diameter, only the 100 μ diameter tube now existed that was in use on the replacement machine. Although circumstantial, there was also evidence that some user had broken the 140 μ tube without recording the fact in writing. It was thought that this occurred at the time of machine replacement.

When these further facts were revealed to the manufacturers they supplied the following formula that would give the factor by which counts made, using the 100 micron counting tube, should be multiplied to give values equivalent to those observed using an aperture diameter of 140 microns.

Correction factor = $\left(\frac{K_1}{K_2}\right)^3$ where K_1 and K_2 are calibration constants for each aperture size.

In this case, $K_1 = 3.5$ for 100 micron diameter

$K_2 = 2.6$ for 140 micron diameter

$$\text{Correction factor} = \left(\frac{3.5}{2.6}\right)^3 = 2.439 \pm 0.073$$

The similarity between this correction and the mean ratio $\left(\frac{\text{cell count at } T = 2.5}{\text{cell count at } T = 21} = 2.480\right)$ described earlier, thus made it highly probable that the difference in counting performance between counters was due to the aperture difference described. This correction factor also agreed well with that derived from apparent cell yield: 2.33 (described in Section 2.3.3).

Although the threshold setting of 2.5 was recognised to be near the lower limit for accurate counts, Coulter Electronics stated that counts at this threshold should still be accurate. Cell concentrations were thus calculated from counts at this threshold rather than counts at $T = 21$ because, being arithmetically greater, they contained less observational error.

As it was only after about a dozen experiments that the altered counting characteristics of the replacement counter became obvious (due to the production of apparently normal steroid production values/cell

due to BSA-related inhibition of steroidogenesis in response to ACTH), these initial experiments contained cell counts obtained at $T = 21$ alone. The following section describes how these counts were corrected so as to be equivalent to readings taken at $T = 2.5$ (and $T = 21$ on the original machine.)

2.3.4.2 The correction of counts made at threshold $T = 21$ on the replacement counter

The ratio of counts at $T = 2.5$ to those at $T = 21$, on the second machine, were averaged for each strain and used to form corrected counts in experiments where estimations had been made at $T = 21$ only.

Table 2.7 gives the mean $T = 2.5:T = 21$ count ratio obtained for the indicated number of individuals for each strain. The arcsin transformations of the means and standard deviations are also shown which enabled parametric statistics to be applied to these ratios.

t test probabilities of differences between arcsin means are given in Table 2.8, expressed as percentages.

It was assumed that significant differences ($p = 5\%$) reflected the strain specific compositions of cell suspensions. The ratios of the lipid replete lines were lower than those of DBA, and backcrosses to DBA, probably because the latter strains were relatively deficient with respect to large lipid-filled cells.

As pooling the ratios for strains, not significantly different in Table 2.8, appeared to increase the variances of most of the distributions containing values so corrected, it was decided that correction should be carried out using individual strain-specific mean ratios.

Table 2.7. Ratio of counts at T = 2.5 to those at T = 21 for each strain.

Strain	Mean($\frac{T = 2.5}{T = 21}$)	No. Individuals	Arcsin Mean	Arcsin Std. Deviation
C57	2.467	16	39.696	1.9844
DBA	2.542	5	38.865	0.8181
CBA	2.247	3	41.864	0.7997
N	2.659	10	37.905	1.4955
O	2.509	14	39.258	1.8177
NC	2.338	17	40.939	1.6239
OC	2.473	16	39.636	1.9767
ND	2.630	21	38.144	1.3893
OD	2.491	22	39.436	1.8328

Table 2.8. t test probabilities of difference between strain specific cell count ratios. (Probabilities expressed as %)s)

	<u>DBA</u>	<u>CBA</u>	<u>N</u>	<u>O</u>	<u>ND</u>	<u>OD</u>	<u>NC</u>	<u>OC</u>
<u>C57</u>	NS	1.0	2.0	NS	2.0	NS	NS	NS
	<u>DBA</u>	1.0	NS	NS	NS	NS	0.1	NS
		<u>CBA</u>	0.1	1.0	0.1	0.1	NS	1.0
			<u>N</u>	NS	NS	2.0	0.1	2.0
				<u>O</u>	NS	NS	2.0	NS
					<u>ND</u>	2.0	2.0	2.0
						<u>OD</u>	2.0	NS
							<u>NC</u>	5.0

(all probabilities two-tailed)

In summary, the ten experiments carried out, after Coulter replacement had occurred, but before a difference in counting had become obvious, were corrected by multiplying the counts (obtained at $T = 21$) by strain-specific ratios ($\frac{\text{count at } T = 2.5}{\text{count at } T = 21}$), observed in later experiments, in which counts at both threshold settings had been carried out.

2.4 ELECTROPHORETIC IDENTIFICATION OF ISOCITRATE DEHYDROGENASE (Idh-1) AND MALIC ENZYME (Mod-1) ISOZYMES

2.4.1 Introduction

Electrophoretic variants have been shown to occur in mice of the supernatant forms of isocitrate dehydrogenase, (Idh-1), (Henderson, 1965) and malic enzyme, (Mod-1), (Shows et al., 1970). The former has been mapped to chromosome 1, and the latter to chromosome 9. (Malic enzyme, although an oxidising enzyme of malate, is distinct from malate dehydrogenase in terms of cofactor, product formed and genetic locus).

As DBA/2J and C57BL/Tb differ in the variants they possess of both enzymes, backcross mice to both these parental types were scored for Idh-1 and Mod-1 phenotypes.

2.4.2 Buffer solutions

2.4.2.1 Gel and tank buffer

Starch gels were made using an approximately 1:30 dilution of the tris-citrate tank buffer which had the following composition:

0.223M Tris base	NaOH added until pH=6.2
0.086M Citric acid	

2.4.2.2 Staining buffer

For Mod-1, this consisted of a 0.2M, and for Idh-1, 0.1M, solution of Tris base/Tris HCl made up according to the manufacturer's mixing table such that, at 37°C, the pH was 8.0.

2.4.3 Preparation of liver homogenates

Liver samples taken from backcross mice, during removal of adrenals consisted of approximately half to two thirds of the liver (depending on size).

Samples collected during dissection were instantly frozen on dry ice and eventually stored at -20°C for about 2 - 3 weeks. After defrosting, homogenisation was carried out, using a plastic pestle rotated at 1,000 r.p.m., in 2 mls phosphate buffered saline. Homogenates were kept on ice until centrifuged at 15,000 r.p.m., for 30 minutes. A little of the clear supernatant was then aspirated off and placed in small storage vials; care being taken to exclude any of the upper lipid layer.

Electrophoresis was nearly always carried out the same day, although samples that had been stored at -20°C appeared to possess identical electrophoretic and staining properties.

2.4.4 Preparation of starch gels and running of samples

Starch gels were formed in 4 x 11 x $\frac{1}{4}$ inch plastic moulds by the conventional technique. Briefly, a 12.5% starch (Hydrolysed starch, Connaught Laboratories) solution in tris-citrate buffer was heated to between 140°C and 150°C, accompanied by agitation to prevent lump formation. Once the solution had boiled for a minute or two and been

briefly allowed to settle, it was poured through a muslin filter into the mould. The surface of the solution was quickly covered by a piece of plastic sheeting, a glass plate, and lead weights. After an hour or two, when the starch was solidified, the gel plate was cleaned of excess gel and covered, excluding trapped air, with a piece of polythene, before storage at 5°C for 4 hours.

Having removed a small piece of gel at the cathode end of the plate, and having bisected the gel with a scalpel into equal halves, the cathode portion was carefully pulled down into the gap to allow loading of the gel to be carried out. Filter paper inserts (5.5 x 8 mm) were uniformly laden with sample and blotted, briefly, on paper tissue to remove any excess. Forceps were washed between each sample. Each gel was loaded with eight samples, consisting, where possible, of four from each parental backcross so that isozyme class could be easily deduced.

At the end of insert placement, the gel was restored to its original state and liquid paraffin was applied to the small gaps between inserts in order to displace air contained within them. Having reapplied the polythene to the gel surface, as before, the loaded gel was placed, horizontally, on supports in a bath of water containing ice cubes, in a cold room (5°C). The level of the water was adjusted so that as much of the plate as possible was submerged. Filter paper, folded four times, was used to form buffer bridges between the apices of the gel and the cathode/anode reservoirs of tris-citrate tank buffer, and also between the dual compartments of each reservoir, in the furthest-most of which were placed the platinum electrodes. Having replaced the polythene over the gel and wicks, electrophoresis was commenced with a constant voltage of 160 volts which was checked in the gel by

inserting needle electrodes, a constant distance (10 cms) apart, and observing the potential difference on a small voltmeter.

This constant voltage was maintained for 17 hours.

2.4.5 Staining of gels

After electrophoresis, the gel was sliced using a thin metal blade and slicing tray.

Cut surfaces nearer the top of the gel were stained for isocitrate dehydrogenase as it travelled about two thirds the way toward the anode, on average, whereas bottom slices were more appropriate for malic enzyme, which travelled only about a quarter of the way to the cathode. (As the anode/cathode wicks were placed on top of the gel, migration of charged proteins tended to occur vertically as well as horizontally.)

2.4.5.1 Stains

The staining reactions were the same for both enzymes; consisting of the precipitation of dye as a result of the reduction of nicotinamide adenosine diphosphate (NADP) with hydrogen cleaved from the substrates; isocitrate and malate. The only major differences between the stains consisted of those of substrate and molarity of the tris base/tris HCl buffer. Both stains contained:-

20 mgms NADP

10 mgms nitro blue tetrazolium

4 mgms phenazine metasulphate

per 100 mls buffer, which was 0.1M tris base/tris HCl for isocitrate dehydrogenase (Id) and 0.2M for malic enzyme (Mod).

Further additions per 100 mls of buffer for each stain were as follows:-

Id	Mod
0.176 gm trisodium isocitrate	0.67 gm malic acid
0.4 ml of a 0.25M solution of $Mn Cl_2$	0.5 ml of a 0.25M solution of $Mn Cl_2$

Stains were stored in dark bottles, at 5°C, for not more than a few days.

2.4.5.2 Isozyme recognition

Starch gel slices were placed in staining dishes of appropriate proportions and after additions of small quantities of stain were incubated, in the dark, at 37°C. Although Id generally took only an hour or so to stain completely, Mod took between 3 - 5 hours.

With both enzymes a single fast or slow band characterised the parental isozymes while three bands, in the case of Id, and one intermediary band, in the case of Mod, were found for heterozygotes. (The intermediary band for Mod really consisted of 5 bands (Shows et al., 1970) but these remained unresolved as the migration of malic enzyme to the cathode was relatively small, under the conditions used.) The presence of two parental, and a more deeply staining intermediary band in Id heterozygotes indicated the simpler dimeric structure of this enzyme as opposed to the probable tetrameric constitution of Mod.

Isozymes were recognisable in three ways: by the number of bands (in the case of Id), the distance travelled (which remained fairly constant between gels) and deduction from the contrasting backcrosses run on the same gel.

2.5 IMMUNO-ELECTROPHORESIS

This technique was used to examine the purity of bovine serum albumin (BSA), for reasons described in Chapter 3, and was essentially the same as the original method described by Grabar and Williams (1953).

By permitting proteins, separated by electrophoresis, to form immunoprecipitates with specific antibodies, high degrees of qualitative resolution are possible using very small amounts of starting protein. The first of these advantages was particularly important to its use in the present work.

Agar plates were prepared from a 2% solution of purified agar (Behringwerke) in 50 mls of buffer and 50 mls distilled water. The buffer was composed of 13.38 gms sodium 2,2-diethylbarbiturate and 8.83 gms sodium acetate trihydrate made up to 1.5 litres with distilled water. The solution was boiled for 15 minutes in a waterbath and 10 mgms of merthiolate were added as preservative. 6 ml aliquots were then added onto alcohol-washed glass plates (10 x 10 cms) and after solidification of the agar, plates were placed in a moist chamber until used.

A plate was loaded for electrophoresis by cutting sample wells in the agar, using Pasteur pipette tip and scalpel, which were placed about 1 cm apart in a vertical line, about one third of the way across the plate from the cathode (BSA travelled almost to the anode, under the conditions described, so greater electrophoretic resolution could be obtained by augmenting the anodal proportion of the plate.) During this and subsequent handlings of the plate, plastic gloves were worn to prevent skin contamination. Two microlitres of each BSA sample solution (15 mgs/1 ml phosphate buffered saline) were placed in the sample wells using a microsyringe.

Electrophoresis was then carried out using barbitone buffer with potential difference of 0.24 volt between the wicks (6 cms apart). This lasted $1\frac{1}{2}$ hours at 5°C .

The next stage, addition of antiserum, was enabled by the creation of troughs (6 cms long and 0.1 cm wide) equidistant between the sample wells and parallel to the electrophoretic axes. 150 μls of rabbit anti-rind serum (cattle) (Behringwerke) were placed in each trough and protein precipitation was then allowed to occur (at right angles to the electrophoretic axes) in the moist cabinet for 24 hours.

After washing in 1 part phosphate buffered saline:9 parts distilled water and replacing the glass plate under the gel, drying was effected by placing in front of a fan for an hour or two, with a piece of blotting paper applied to the gel surface.

After staining in 2% Coomassie Blue for half an hour, destaining in 50% methanol, 7% acetic acid occurred until maximum clarity was obtained.

2.6 DRIED ORGAN WEIGHTS

In relating the size of certain organs to the observed in vitro adrenal function it was necessary to measure dry weights as time restrictions, on the day of adrenal incubation, prevented the fresh organs from being dissected out immediately. Accordingly, immediately after removal of adrenals, corpses were frozen at -20°C , and dissected at a later date. Corpses from 4 - 6 experiments were usually dissected at once so that the periods during which they had remained frozen ranged from a few days to about 10 weeks. Any differential decomposition and dissociation into volatile products such as carbon dioxide, water or ammonia was not appreciable, either by comparison of organ appearance or dry weight.

Dry weights were obtained by weighing aluminium foil strips, placing dissected organs onto these and reweighing, after they had been dried in an 80°C oven until weight remained constant over 3 weeks. The period required to obtain this constancy was about a month.

Weights were measured to the nearest 50 micrograms.

2.7 COAT COLOUR MARKER GENES

The segregation of the dilute allele (d), the brown allele (b) and the non-agouti allele (a), on chromosomes 9, 4 and 2, respectively (Green, 1966), was studied in backcrosses to DBA which is recessive for all three of these genes; being dilute, brown and non-agouti.

2.7.1 Backcrosses of C57 x DBA F₁s to DBA

C57 mice are both concentrated (D) and black (B) so four equal classes of the following type were obtained, although suggestive disproportion between these classes was observed:

<u>Phenotype:</u>	Black	Brown	Grey	Light brown
<u>Genotype:</u>	Dd,Bb	Dd,bb	dd,Bb	dd,bb

2.7.2 Backcross of CBA x DBA F₁ to DBA

The presence of agouti (A) as well as concentrated (D) and black (B) in CBA mice, resulted in 8 segregant classes of equal size:

<u>Phenotypic description</u>	<u>Genotype</u>
Black agouti (identical to CBA)	Dd, Bb, Aa
Black (identical to C57)	Dd, Bb, aa
Pale sandy brown (pale CBA-like)	Dd, bb, Aa

<u>Phenotypic description</u>	<u>Genotype</u>
Plain dark brown	Dd, bb, aa
Agouti pale grey	dd, Bb, Aa
Grey	dd, Bb, aa
Very light brown (paler than DBA)	dd, bb, Aa
Light brown non-agouti (identical to DBA)	dd, bb, aa

Normally, scoring these segregant classes was carried out without difficulty but when any doubt existed, particularly in distinctions between agouti and non-agouti light colours, a second opinion was sought and the original scoring, in every case, was upheld.

CHAPTER 3

THE ADRENAL DISSOCIATION AND INCUBATION TECHNIQUES3.1 INTRODUCTION AND HISTORY OF ADRENAL DISPERSION3.1.1 Introduction

The use of tissue slice incubations in the study of steroidogenesis by adrenal cells has already been discussed. The deficiencies of this technique have been outlined and reasons have been shown for favouring an isolated cell (or cell suspension) method.

Isolated cells have been produced for many years for diverse purposes such as formation of primary cell cultures and production of tumour cell inocula. This has resulted in a variety of methodologies utilising varying severities of enzymic digestion dependent upon the amount of starting tissue, the resilience of differing cell types to such dispersion and the importance of the need to preserve the natural state of the cell by avoiding excessive cell membrane damage.

Where cell culture techniques have been used to study the adrenal, differentiation of a primary foetal cell line into mature differentiated cells, capable of steroidogenic responses, has been effected by meeting certain growth requirements including the presence of ACTH (O'Hare and Neville, 1973; Milner and Villee, 1970; Milner, 1971; Milner, 1973).

An isolated cell technique assumes that, provided cell dissociation is not so disruptive as to require an extended culture period in order that reversible damage to a differentiated response be repaired, it should be possible to study unimpaired cell functions in the primary cell suspension. If many different individual cell lines are to be analysed, a mandatory feature of even the simplest genetic studies, great economies can be made

with respect to time and space. The possibility also exists that it might be advantageous to subject certain cell types such as endocrine cells to as little of the in vitro environment as possible in order to obtain as authentic a model of the in vivo situation as possible. The assumption, of virtually unimpaired response, can be placed at risk if the desire to obtain high proportional cell yields is not matched by an awareness of the potential cell damage wrought in order to obtain that end, especially when the amount of starting tissue is very small.

Most isolated cell experimentation has accordingly employed primary tissue derived from large and medium sized mammals so that studies on the pituitary, adrenal and other small organs have not been hampered by cell yield considerations.

A major part of the present work has consisted of scaling down dispersal techniques such that mouse adrenals, rather than those from other, less extensively studied, mammals, can be utilised, ultimately to the extent of studying the steroidogenic response of cells derived from the single pair of adrenals from an individual mouse.

The three major sections of this chapter describe, firstly, in Section 3.2, the method for preparing isolated cells from pooled adrenals and the mechanical and enzymic modifications required to adapt it for single organ pair dispersion. Section 3.3 provides evidence that impurities in bovine serum albumin (BSA) batches were responsible for inhibiting ACTH-mediated corticosterone production in some experiments. Also described is the partial characterisation of the impurities by means of immuno-electrophoretic separation. A concluding section indicates the merits and limitations of the dispersion and incubation procedures.

3.1.2 The History of the Isolated Adrenal Cell Technique

The need for a reliable bioassay of ACTH, before radioimmune techniques were widely used, acted as the initial incentive to disperse adrenal tissue to give cell suspensions that could be incubated, immediately, in short-term culture.

Although the basic components of methods were similar in consisting of mechanical disruption, either accompanying or following enzymic digestion of the inter-cellular matrix, the fine details, such as the enzymes and mechanical agitators used, were varied. The source of adrenal tissue has been almost invariable, however; the rat, with its superficial advantage of size, has been the rodent of choice. Although dispersion methods, originally designed for bioassay, began to be used to study adrenal function, no attempt has apparently been made to study mouse adrenals, in this way. The ensuing description of different dispersion methods therefore applies only to rat adrenals.

An original technique using collagenase (Kloppenborg et al., 1967) with magnetic stirring was only partially successful as a proportion of dispersions produced relatively unresponsive cells. It was suggested that a contaminant of the collagenase might have accounted for this. Although this is a plausible explanation, incubations using collagenase in the present work indicated the likelihood of such variability being caused by impurities in the bovine serum albumin used, as will be shown shortly.

A protocol clearly designed to satisfy the demands of a neat bioassay, used trypsin in conjunction with magnetic stirring (Swallows and Sayers, 1969). What the method achieved in good yields of highly responsive cells it appeared, like Kloppenborg's method, to suffer from incomplete reproducibility, bearing out the hypothesis that something other

than collagenase contamination was responsible. In a later, improved, method (Sayers et al., 1971), replacing the magnetic stirrer with a glass paddle, but still using trypsin on its own, the same group found bovine serum albumin to be necessary to yield responsive cells but observed that sensitivity to ACTH declined at albumin concentrations higher than 0.5%. Trebling the calcium concentration was also found to increase sensitivity. The use of extreme ion or metabolite concentrations to create hypersensitive cells is permissible in the context of bioassays using large adrenal pools derived from the same stock of rats. When the object of study is 'normal' adrenal function, such procedures could be misleading, particularly in comparisons between genetically different strains.

Another original technique involved the use of trypsin and collagenase sequentially (Halkerston and Feinstein, 1968), and this method was characterised with respect to the effects of altering enzymic and mechanical components on cell yield and sensitivity (Barofsky et al., 1973). Other innovations in this method included the use of ribo- and deoxyribo-nucleases to hydrolyse highly viscous material, of nuclear origin, that tends to impede the histolytic enzymes. Mechanical disruption was performed by excursion through Pasteur pipette rather than by continuous stirring.

Apart from being the most clearly characterised technique, the procedure advocated by Barofsky was felt to be founded on a sounder objective than other methods, namely: the dispersal of cells brought about by the minimum of enzymic and mechanical interference. This was achieved by considering the biochemical nature of the intercellular matrix, and polymers more intimately associated with each cell.

The increased enzymic histolysis, accomplished by using trypsin and collagenase sequentially, has the additional advantage of lessening the

amount of mechanical agitation required to disperse tissue fragments into single cells. This succeeds in reducing mechanically-induced cell damage, and also the time period during which isolated cells are exposed to histolytic enzyme. On the basis of these considerations the Halkerston and Feinstein technique, as reappraised by Barofsky, was considered a suitable master-plan from which to develop the mouse adrenal dispersion method, described in the following sections.

3.2 MOUSE ADRENAL DISPERSION AND ACTH INCUBATION

On the basis that rat adrenals are approximately five times the size of mouse adrenals, the rat dispersion methodology (Barofsky et al., 1973) was scaled down by one fifth.

3.2.1 Composition and Preparation of Buffers and Stock Solution

Krebs-Henseleit original Ringer bicarbonate buffer was made up from the component salt solutions whose concentrations are expressed as % or gms/100 mls:

<u>% (gms/100 mls)</u>	<u>Salt</u>	<u>Parts</u>
0.9	NaCl	100
1.15	KCl	4
1.22	CaCl ₂	3
2.11	KH ₂ PO ₄	1
3.8	MgSO ₄ ·7H ₂ O	1
1.3	NaHCO ₃	21

The NaHCO₃ was bubbled with CO₂ for an hour before adding to the other salts.

0.2 gms/100 mls buffer of glucose were added to the made up buffer to give Krebs Ringer bicarbonate with glucose or KRBG. The pH of freshly prepared KRBG was raised from pH 6.2, initially, to a working value of between 7.15 and 7.30, by gassing with 95% oxygen/5% carbon dioxide (95% O₂/5% CO₂) for 30-40 minutes. The other major buffer used, a 4% solution of bovine serum albumin (Sigma Chemicals, fraction 5, essentially fatty acid free) in KRBG, was prepared by allowing dissolution of the protein to occur, standing at 4°C, beneath 95% O₂/5% CO₂, in KRBG at the working pH. This was to avoid any fluctuation in pH caused by excessive agitation of the buffer and also to minimise frothing which might cause the final volume to be reached inaccurately.

Both KRBG and the 4% albumin solution (KRBG-BSA) were made up the day prior to experimentation and the buffers were always pH-ed before storage at 4°C under 95% O₂/5% CO₂.

If a buffer showed a pH slightly outwith this range, its bottle was gassed with either 95% O₂/5% CO₂ or 100% CO₂ and then agitated for a few seconds.

Any solution that had a markedly abnormal pH (which could not be normalised in the manner described) was discarded and made up afresh. In these rare instances it was assumed that an error had been made in the addition of one of the salt solutions.

Salt stock solutions, at five times normal concentration, were deep frozen and thawed when required, in order to decrease the chance of weighing error. Such stocks were free of microfloral contamination for virtually unlimited periods.

ACTH stock solution

The manufacturer advised that ACTH (Sigma porcine grade II) could be maintained in a stable state for months in saline acidified with HCl, to pH 4, at 4°C.

It was found, in the present work, that solutions of 1 mgm. ACTH (approximately 88 international units or I.U.s) in 100 mls of acidified saline (pH 4), kept at 4°C, remained potent for 5 weeks. This was the maximum period for which they were kept although microbial growths were not visually evident until about 2-3 months.

A comparison between identical solutions, one kept at 4°C, the other frozen at -20°C, revealed a loss of potency in the latter. This form of storage was, thus, never used.

3.2.2 Pooled adrenal dispersion and ACTH incubation

Each mouse was treated in the following way, always between 9.45 a.m. and 11.15 a.m.

The cage, usually containing a moderate number (i.e. 2 - 4) (see Chapter 2 for conditions of caging), was opened as quietly as possible and the most excited mouse always used first on the basis that it could stir up alarm amongst its remaining cage-mates. The mouse was then dispatched, by cervical dislocation, within five seconds of removal from its cage. Its body weight was then noted to the nearest 0.025 of a gram.

Adrenals were then dissected out, ventrally, using fine forceps and 3.5 inch iris scissors. After clearing the organs of fat tissue, each adrenal was bisected, using the scissors, along its longest axis, and finally placed in a siliconised, 25 ml, Erlenmeyer flask, containing Krebs Ringer bicarbonate buffer containing 0.2% glucose (KRBG), which was replaced on ice. As the corpses of the mice were stored at -20°C until

a future date, when other organs were removed, each mouse was tagged with its number in order of dissection.

(All glassware used was siliconised using the method outlined earlier.)

After all adrenals had been collected, the first of the two enzymic incubations was commenced. KRBG was aspirated off from the collection flask and was replaced with 0.4 ml per adrenal pair of KRBG containing the freshly dissolved enzymes; 0.25% bovine trypsin (Sigma type 3), $1.25 \times 10^{-3}\%$ deoxyribonuclease (Sigma, bovine type 1) and $5 \times 10^{-3}\%$ ribonuclease (Sigma, bovine 1-A). This usually consisted of 4 mls of the above solution as 10 adrenal pairs were the usual number in pooled dispersions. (The DNase and RNase were included to prevent inhibition of proteolysis, as mentioned earlier). When two pools of about five adrenal pairs, from different strains of mice, were being compared, it was found that 25 ml flasks were still suitable to take 2 mls of trypsin buffer each. The flask was then thoroughly gassed with 95% O₂/5% CO₂ and incubated at 37°C, in a shaking waterbath (64 oscillations per minute) for 1 hour.

At the end of this incubation, the trypsin buffer was aspirated off and replaced with 0.2 ml per adrenal pair of KRBG. The adrenal bisects were washed a further two times with similar volumes of KRBG containing 0.05% of the trypsin inhibitor, ovomucoid (Sigma, type 2-0).

The second incubation was carried out in a fresh flask containing 0.2 ml per adrenal pair of KRBG-BSA (KRBG with 4% bovine serum albumin), containing the same concentrations of deoxyribonuclease and ribonuclease as before and 0.02% chromatographically purified collagenase (Sigma, type 3A from *C1. histolyticum*). After gassing with 95% O₂/5% CO₂,

incubation was commenced at 100 oscillations/minute for one hour.

Ten minutes prior to the end of the incubation period, the dispersion process was carried virtually to completion by aspirating the buffer and adrenal fragments, gently, into a Pasteur pipette (approximately 1.2 mm diameter) and ejecting; the complete excursion being carried out 70 times. The suspension was then incubated for a further 8 minutes.

The crude suspension was then filtered through two wire mesh filters of differing mesh (30 spaces/cm. with 178 micron openings, and 80 spaces/cm. with 74 micron openings). The filtration was carried out by withdrawing the suspension from the incubation flask, into a 1 ml syringe. The contents were then, slowly, expelled through two plastic filter holders that fitted onto the syringe, and onto each other, so that the double filtration was performed simultaneously.

A new filtration unit, consisting of holder and mesh trimmed to form a snug fit in the filter recess, was used for each strain and each experiment.

The filtered suspension, consisting of single cells and small aggregates, was collected in 15 ml Pyrex centrifuge tubes so that not more than 0.7 ml was placed in any tube.

The first centrifugation was carried out at 4°C after balancing the load, using a balance. After 20 minutes at 200g, the collagenase buffer was aspirated off and replaced by 0.7 ml per tube of KRBG-BSA. Centrifugation was repeated, as before, after careful resuspension of the cells using a siliconised Pasteur pipette.

The first KRBG-BSA wash was replaced with another and, before the third, and final, centrifugation, 0.05 ml of suspension was removed and

placed in a dilution of saline for counting, using a Coulter counter. This was only done when more than one suspension was being prepared so that by appropriate adjustment of the final resuspension volume, in KRBG-BSA, the concentration of cells was roughly the same for each strain. The Coulter counter setting was such that probably all cell types, except blood cells, were counted thus corresponding to results of total adrenal cell counts obtained using an haemocytometer. The use of a replacement Coulter counter with apparently different counting characteristics required a new threshold setting to be adopted, as has been described. Nearly all pooled experiments used the original machine, however.

The final resuspension in a minimum of 0.7 ml of KRBG-BSA included the addition of a further amount of buffer to the suspension with the higher Coulter count, in the case of a dual strain comparison, such that the final cell concentrations were about the same.

The ACTH incubation vials (6 dram vials; 25 x 60 mm with snap-on-plastic caps) already contained 0.3 ml each of KRBG-BSA and 0.005 ml of acidified saline (vehicle alone) or 0.005 ml of a dilution of ACTH in acidified saline. These were generally prepared the day before, when the buffers were made up, and were stored at 4°C under 95% O₂/5% CO₂.

To the contents of these vials were added 0.3 ml of the final suspension; each strain or treatment being duplicated. The total incubation volume was 0.605 ml and after gassing with 95% O₂/5% CO₂, the steroidogenic incubation was carried out for one hour with 64 oscillations per minute, at 37°C.

Of the remaining suspension, 50 µl was removed for a second Coulter count, and the rest was centrifuged at 100g for 30 minutes and then subject to viability determination.

Viability was assessed by the common dye exclusion method that utilised a solution of 0.24% trypan blue and 0.85% NaCl. After aspirating off any remaining buffer, 0.2 ml of the trypan blue solution was added and cells were gently resuspended. The proportion of unstained cells, comprising the total counted, was observed after 10 minutes.

The ACTH incubation was arrested by the addition of 39% ethanol to the vials which were then stored at -20°C until analysed for corticosterone.

3.2.3 Modifications used to disperse individual adrenal pairs

Early single adrenal pair dissociations did not display the high degree of dispersion, observed in pooled adrenal experiments, when a scaled-down replica of the pooled technique was used. Retrospectively, this was not a serious problem as enough cells were harvested to produce easily analysable concentrations of corticosterone. At this time, however, an uncontrolled factor (bovine serum albumin purity) was causing many of the suspensions to be unresponsive to ACTH. It was concluded, after making the obvious checks on buffer composition and pH, that this inhibition was the result of some collagenase contaminant which had a more marked effect when cell yield was low, although some of the earlier pooled adrenal incubations displayed inhibition, although less commonly. It seemed expedient, therefore, to maximise cell yield and also find a batch of collagenase that had no inhibitory effects and obtain a sufficient amount of it to complete the study.

The first objective was only partially successful while the latter, by its failure, indicated that inhibition was being exerted by a contaminant in one of the other reagents. The eventual elucidation of the true contaminant (see Section 3.3) abrogated the need for further improvement in cell yield.

These two separate experiments each employed 4 pairs of adrenals such that half an adrenal from each pair formed one suspension. ACTH incubations were unduplicated.

These suggestive data bore out expectations. Increased trypsin concentrations were anticipated as having little effect on the ACTH-responsiveness of cells still associated in tissue conglomerates. Increases in collagenase concentration have been found to depress the responsiveness of cells to 3', 5' - cyclic adenosine monophosphate (cAMP) (Barofsky et al., 1973) but calculation from their original data indicates less than a 10% drop when the concentration is raised to 0.05%.

In contrast to earlier findings (Barofsky et al., 1973), the addition of 0.05% of the mucolytic enzyme, hyaluronidase (Sigma, from bovine testis type 1), to the collagenase incubation was found, in a larger experiment employing 16 adrenal pairs, to increase cell yield.

The adrenal pairs were distributed randomly between 8 pools (2 adrenals each) which were dispersed using three variables:

- (1) 0.05% hyaluronidase in the collagenase incubation.
- (2) Pure or crude collagenase.
- (3) Collagenase incubation volume.

The cell yields (given as original Coulter counts) for the various treatments are shown in Table 3.2.

Dispersion did not appear to be raised by using crude collagenase, but it was by inclusion of hyaluronidase and also by larger incubation volume. The latter result was not so marked as to justify the use of this incubation volume but it held certain mechanical implications which will be discussed in due course.

The following sub-sections contain descriptions of the enzymic and mechanical modifications of the original pooled technique that were adopted to disperse individual pairs of adrenals.

3.2.3.1 Enzymic modifications

The concentrations of the two major enzymes were increased such that 0.35% trypsin (1.4 x original) and 0.04% collagenase (2 x original) were the new concentrations.

The data shown in Table 3.1, obtained from two separate experiments are too small to form a statistically sound judgement. They do, however, form good circumstantial evidence that neither enzyme concentration increase resulted in significant distortion of the ACTH response. Two variables were examined in each experiment.

Table 3.1 Relationship of enzyme and calcium concentrations to cell yield and steroidogenic capacity. (R = pgms corticosterone produced/viable cell/hour).

Experiment 1			
0.5% Trypsin		0.25% Trypsin	
Cell yield/ml	R	Cell yield/ml	R
244,800	1.78	238,400	1.78
283,000*	1.99	226,000*	1.80
Experiment 2			
0.08% Collagenase (4 x normal conc.)		0.04% Collagenase (2 x normal conc.)	
Cell yield/ml	R	Cell yield/ml	R
305,000**	1.96	210,000**	2.13
230,000	2.15	240,000	2.16

* Calcium-free collagenase incubation medium.

** Dispersed using a steel ball bearing, as described shortly.

Table 3.2. Effect of collagenase concentration, volume and hyaluronidase on cell yield.

0.05% hyaluronidase; collag. volume (ml);	0.04% pure collagenase				0.3% crude collagenase			
	With		Without		With		Without	
	0.3	0.6	0.3	0.6	0.3	0.6	0.3	0.6
	1484	2205	1306	1451	1012	1866	1129	1255
	1374	2063	1035	1082	1247	1604	1281	1478

A three-way analysis of variance revealed the following significant effects:

Treatment	P	Interactions	P
Collagenase purity	N.S.	Collagenase x hyaluronidase	0.05
Incubate volume	0.01	Incubate vol.x hyaluronidase	0.05
Hyaluronidase	0.01		

In order to ensure that hyaluronidase treatment did not significantly alter the steroidogenic response, the responsiveness of cells, dispersed with, and without, the enzyme, was examined.

The effects of carrying out two collagenase incubations, of half an hour each, were also studied. (This involved carrying out the first mechanical dispersion, aspirating off the cells released and reincubating with fresh collagenase, ending with another dispersion.)

Six C57 adrenal pairs were used in pairs so that each of two suspensions (hyaluronidase and no hyaluronidase treatment) in a row were comparable in that they were formed from identical adrenals (see Table 3.3.).

Table 3.3. Effects of double collagenase incubation and hyaluronidase on cell yield and responsiveness.
(R = pgms corticosterone produced/viable cell/hour.)

Adrenal Group	0.05% Hyaluronidase		No Hyaluronidase	
	Yield/ml	R	Yield/ml	R
1	434,000*	3.44	353,080*	3.70
2	364,280*	3.39	300,720	3.46
3	364,000	5.97	331,240	5.34

* Produced by double collagenase incubation.

(ACTH incubations were duplicated for each suspension.)

Comparisons within identical adrenal pair groups indicate that hyaluronidase produces no significant departure from normal dispersion with regard to steroidogenic response, a result that concurs with Barofsky et al., 1973.

Double collagenase incubation also raised cell yield, slightly, but was found to be impracticable, when about 12 adrenal pairs were being separately dispersed, due to time factors.

As calcium-free incubation media appeared to have little effect on cell yield and may have altered the responsiveness of cells to ACTH (Table 3.1), normal concentrations were used, according to the KRBG formulation, described already.

3.2.3.2. Mechanical modifications

Changes in mechanical aspects of the technique, (apart from those solely relating to the process of dissociation, described in Section 3.2.4) were related to those parameters assisting enzyme permeation

of tissue prior to dissociation. Adjustments within this category were all resultant from a common cause; the reduced inertias of smaller incubation volumes. Agitation of tissue within incubation buffers by means of the shaking mechanism of the waterbath (to and fro along a linear path) requires a certain level of inertia (proportional to mass or volume of incubate), to be effective.

The results of the experiment referred to earlier (Table 3.2) indicated that using 0.6 mls, instead of 0.3 mls, as collagenase incubation volume increased cell yield. This could be partly due to reduction in enzyme inhibition by products of lysis but was more probably due to better enzyme permeation of tissue fragments as a result of the greater inertia of the larger volume.

A number of solutions to the problem presented themselves, the most obvious being:

1. to shake smaller volumes at higher speeds.
2. to keep volumes as large as space allowed.
3. to use adrenal fragments rather than bisects in the collagenase incubation.
4. to introduce an object with high inertia (a small ball bearing) into one, or both, enzyme incubations.
5. to apply more force to tissue fragments during the final dissociation procedure, using Pasteur pipette of narrower gauge and/or increasing the speed of excursions and/or carrying out more excursions.

These measures were embodied in the modified technique except for the inclusion of ball bearings, which probably increased yields significantly, but also depressed viabilities, as indicated by the data in Table 3.4, obtained from an experiment using four adrenal pairs, distributed as before.

Table 3.4. Effects of including a ball-bearing during dispersion on cell yield, viability and responsiveness.
(R = pgms corticosterone produced/viable cell/hour.)

Ball-bearing (both enzyme incubations)			No ball-bearing		
Yield/ml	Viability	R	Yield/ml	Viability	R
205,600	62.5	3.26	122,800	67.7	2.82
158,400	61.0	3.19	142,800	73.7	4.75

(The ACTH incubations were unduplicated.)

These mechanical considerations resulted in the following incubation conditions being adopted:

- (a) Trypsin incubations had 1 ml buffer/adrenal pair and were agitated at the faster speed of 85 oscillations/minute.
- (b) Collagenase incubations contained 0.25 ml buffer/adrenal pair and were shaken at the faster speed of 140 oscillations/minute.

The trypsin incubation volume was thus increased by 2.5 per adrenal pair. The collagenase volume, conversely, remained roughly equivalent to pooled dispersions (0.2 ml/adrenal pair). This ensured that cells were dissociated at a similar concentration to pooled experiments. It was also used because there was evidence that a smaller volume would tend to increase the efficiency of dissociation due to an increased shearing effect exerted by tissue fragments on themselves as they competed for entry into the Pasteur pipette.

This advantage was probably masked in the experiment revealing increased cell yields from larger collagenase volumes due to imperfect enzyme permeation in the smaller volumes. This experiment was carried

out at the old shaker speeds (64 and 100 oscillations/min) and had it been carried out at higher speeds the yields might not have been significantly different.

The surface area of adrenal tissue was increased prior to collagenase incubation to facilitate enzyme permeation. This was carried out by slicing each adrenal bisect along its longest axis, 3-4 times, using a razor blade.

The bore of pipette used for dissociation was approximately 0.9 mm as opposed to the 1.2 mm used in pooled experiments. A trial was carried out to see if syringe needles with bores of approximately 0.8 mm would be more effective at cell dissociation. They were, in fact, less efficient and may have produced damaged cells, as reflected in the yield and steroidogenesis data contained in Table 3.5. Four C57 adrenal pairs were used, as before.

Table 3.5. Comparison of cell yields, viabilities and responses using syringe needles and pipettes to dissociate cells.
(R = pgms of corticosterone produced/viable cell/hour.)

Dissociated using syringe needle			Dissociated using pipette		
Yield/ml	Viability	R	Yield/ml	Viability	R
177,600	63.2	1.39	250,000	60.0	1.51
216,800	50.8	1.39	255,200	62.5	1.47

(ACTH incubations were not duplicated.)

When very narrow guage pipettes and needles were tried, capillary forces tended to disable speedy dissociation and were also prone to blockage by tissue fragments.

An optimum aperture of pipette appeared to be about 0.9 mm.

3.2.4 Mechanised dissociation

The dissociation of adrenal bisects, by excursion into a Pasteur pipette, could be performed manually, in pooled experiments, because only two, at the most, separate suspensions were required to be produced. In studying up to twelve separate adrenal pairs, however, it became necessary to develop some method that would result in time-saving, simultaneous dissociation, which would have the advantage of subjecting each separate organ pair to a similar degree of mechanical trauma.

A simple piece of apparatus, constructed to perform this task, will be described in the following sub-section, 3.2.4.1.

The other sub-section will give an account of the dissociation protocol using this apparatus.

3.2.4.1 Apparatus for mechanised dissociation

A battery of (up to twelve) Pasteur pipettes, held by metal clips, screwed linearly onto a wooden support, was fed by a manifold of plastic tubing, branched and connected using 'T' and 'Y' pieces and ultimately connected to large rubber bulb. Tubing directly leading to the pipettes was composed of high quality rubber to facilitate pipette removal or exchange.

Early experiments, employing the manifold on its own, with manual compression of the bulb were precarious, even with six adrenal pairs, as it was difficult to repetitively judge the exact compression required to suck most of the buffers into the pipettes without, explosively (through the admittance of air) drawing up too much.

This problem was overcome by constructing, using Meccano, a simple machine that converted a rotary drive into reciprocating motion

used to compress and release, cyclically, the rubber bulb, attached to the manifold. One of the major components of this device, schematically represented, not to scale, in Figure 3.1, consisted of an adjustable collar on the axle bearing leverage, which, by movement along the axle, could be adjusted to give the desired nett throw. (When large alterations in throw settings were made, the collar on the piston axle, bearing the pivot, had to be repositioned, also.)

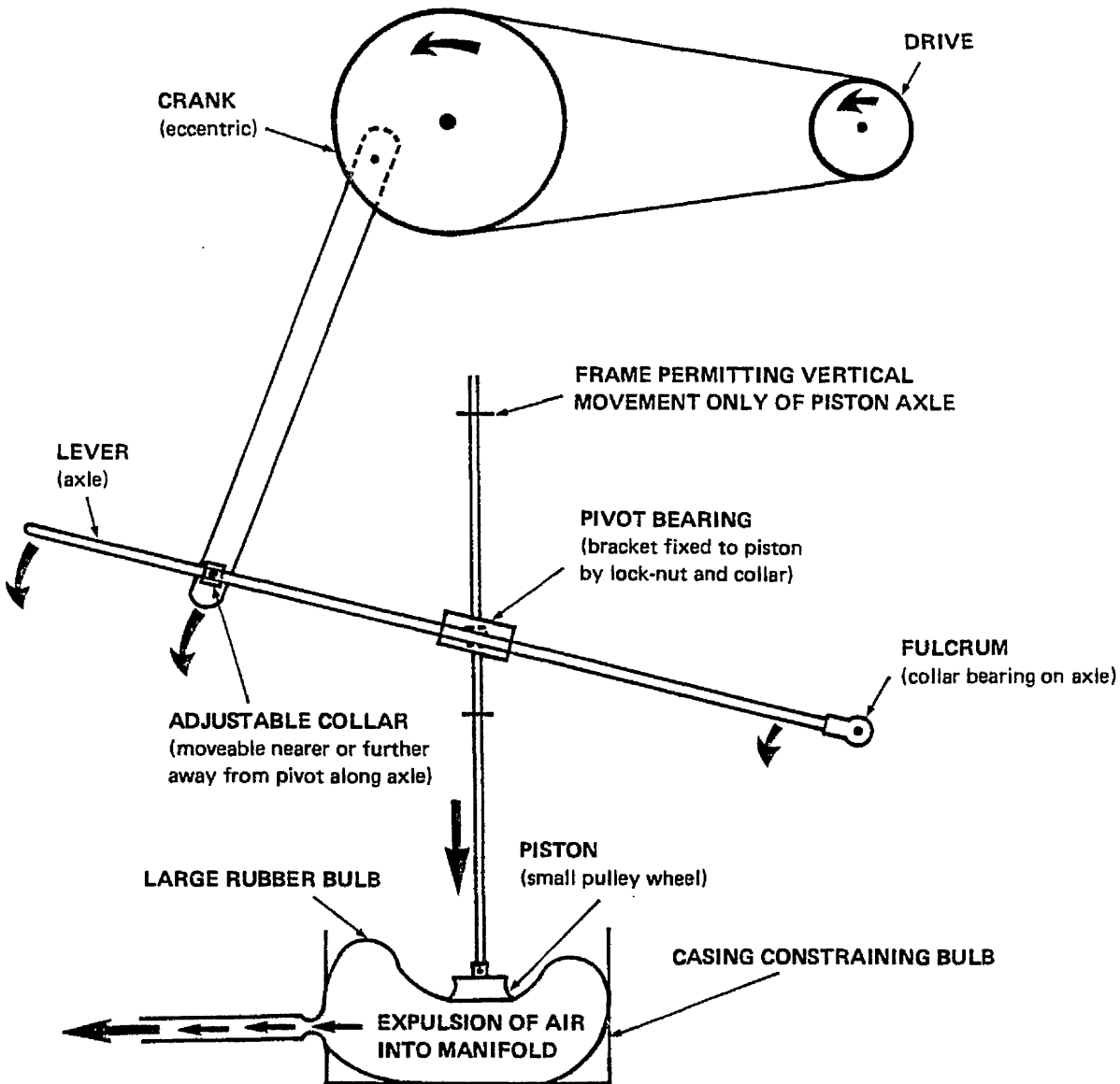
Drive was supplied from an electric motor, powered by nine 1.5 volt batteries, which had a six-speed gear-box. As the battery power pack possessed a switch causing electricity output to be halved, there were, altogether, twelve speeds that could be used, ranging from 14 cycles/minute to 150 cycles/minute. Minor speed alterations were effected by replacing batteries with ones of different electromotive strength.

For most experiments, two dissociation speeds were used for differing lengths of time. It was thus reasoned that one of 55 cycles/minute, for an initial period of one minute, would break larger adrenal fragments into smaller ones, while a further nine minutes dissociation, at half that speed (27.5 cycles/minute), would complete the dissociation into single cells.

The object of this was to maximise yield in a way thought to be least noxious to most of the dissociated population of cells. The constancy of these speed settings, maintained to within ± 1 fast cycle/minute, in the face of battery power decay, was observed during testing of the complete dissociation assembly, on the day preceeding each experimental one.

The trial also ensured the hermetic integrity of the apparatus and other defects, such as imbalanced withdrawal of fluid, by the Pasteur

Figure 3.1 Principal components of dissociating machine with Meccano parts used shown in brackets, (air expulsion cycle nearly complete).



pipette linked to the manifold (usually due to bore differences) were rectified.

As well as being reliable enough to be left, unattended, while the nine minutes dissociation time was used, profitably, elsewhere, the dissociation machine served to lessen inter-experimental error; being a source of objective and invariable mechanical force. Thus, even if an identical force were to have different effects on adrenal cells of different genotypes, provided the force remained constant throughout all experiments, genetic segregation controlling R value would still remain observable, although distorted by such an interaction.

3.2.4.2 The mechanically assisted dissociation procedure

Apart from mechanisation, the other major difference from pooled adrenal dissociation was the collagenase incubation volume. The probable mechanical, rather than enzymic, cause of increased cell yield, obtained using large collagenase volume (see Table 3.2), combined with the high cost of pure collagenase and inconvenience of large volumes, resulted in the adoption of 0.25 ml as the incubate volume.

Dissociation was carried out in narrow (5 x 50 mm) test tubes rather than the broader collagenase incubation vials (15 x 55 mm) for two main reasons:

1. to concentrate the heavier tissue fragments around the dissociating pipette tip, at the end of each expulsion phase of the cycle, so that they would experience as much mutually-effected shearing stress as possible, as they were drawn into the pipette, during the influx phase.
2. to reduce the surface area of buffer exposed to air, rather than 95% O₂/5% CO₂, and thereby minimise any rise in pH.

The protocol followed, when dissociating single adrenal pairs, mechanically, is described in the remainder of this sub-section.

At the end of 50 minutes collagenase incubation, the 0.25 ml

incubates were transferred, using siliconised pipettes, to the small (5 x 50 mm) test tubes. These were then clamped in a line, within a supporting frame lined with foam rubber, so that they were half immersed in a small waterbath, maintained at 37°C. The frame (constructed from Meccano) permitted easy manipulation of the dispersion tubes, gripped firmly, but not too tightly, between its opposing foam rubber sides, thus facilitating the orientation of the tubes with respect to the manifold of Pasteur pipettes.

The battery of pipettes was then lowered so that each pipette tip was 1 - 2 mm off the bottom of each test tube.

Fast dissociation (55 cycles/minute) was then carried out for 1 minute (fresh collagenase buffer was added to any incubate that appeared in danger of being overdrawn, using a syringe).

After reducing the throw of the piston in the machine slightly, (by moving the adjustable collar to a pre-marked spot on the lever axle), 9 minutes of slow dissociation at 27.5 cycles/minute were begun.

Filtration of cell suspensions, at the end of this period, involved the use of modified filters. These consisted of 5 mm lengths of plastic tubing with wire mesh (the finer grade only, of 80 apertures/cm. was used) fused by heat across one end. The cuffs of tubing were then pressed onto 1 ml syringes so that a space of about 1 mm remained between the orifice of the syringe and the wire mesh.

Dissociated cells and tissue fragments were then transferred to syringes (with filters), devoid of plungers and resting on the rims of 15 ml centrifuge tubes.

After replacing the syringe plungers, filtration was very gently completed by pressing the plungers. If a suspension appeared clumpy at a later stage (a rare occurrence, especially when BSA purity was eventually controlled) filtration was repeated using a new syringe and filter.

Filtered suspensions were centrifuged immediately at 4°C. The time taken from the start of dissociation to reach this stage was about 20 minutes.

3.2.5 Complete protocol for the dispersion and steroidogenic incubation of single adrenal pairs

All enzymes were identical to ones used in pooled experiments; being from the same manufacturers and of the same quality. The method of killing experimental mice and their dissection was identical to that of pooled animals, except that liver samples were taken from segregating generations, for Idh-1 and Mod-1 electrophoresis. The adrenal colours (from lipid depleted red to the replete yellow) and coat colours were also observed for these mice.

After removal of the small amount of KRBG bathing adrenal pairs, the six dram vials were given 1 ml each of KRBG containing 0.35% (1.4 x pooled concentration) trypsin, $1.25 \times 10^{-3}\%$ DNase and $5 \times 10^{-3}\%$ RNase.

After gassing with 95% O₂/5% CO₂, trypsin incubation was carried out with a waterbath shaker speed of 85 cycles/min., at 37°C, for one hour.

The initial wash with KRBG, after removal of trypsin from the vials, was omitted on account of the reduced tissue mass and the use of relatively larger volumes of 0.05% ovomucoid KRBG washes; each adrenal pair being washed, twice, with 1 ml of inhibitor.

Adrenal bisects were aspirated into a Pasteur pipette, along with a little of the second ovomucoid wash, and placed, in a small pool of buffer, on a white, glazed tile. Using a razor blade (two edged), previously cleaned of wax (used to retain paper wrapping) using ethanol, each adrenal bisect was sliced 3-4 times along its longest axis.

The tissue fragments were next placed in 15x55 mm. vials, containing 0.25 ml KRBG-BSA with 0.04% collagenase, DNase and RNase as before, and 0.05% hyaluronidase. After 50 minutes of incubation, shaken at 140 cycles/minute, the contents of each vial was transferred to a small test-tube (5x50 mm) for mechanical dissociation, as described already, in more detail, in Section 3.2.4.2.

Filtered suspensions were then centrifuged at 200g at 4°C for 20 minutes, after which the enzyme supernatants were replaced by 0.5ml KRBG-BSA. After very gentle resuspension, using siliconised Pasteur pipettes, the next centrifugation, identical to the first, was carried out.

Resuspension, the second time, was performed in 0.65ml KRBG-BSA and before the final centrifugation, under the same conditions, 0.05ml of each suspension were removed for saline dilutions which were Coulter counted. Cells were resuspended, for the last time, in a minimum of 0.55ml KRBG-BSA, with appropriate additions of buffer to equalise cell concentrations, as indicated by the first Coulter counts.

The ACTH incubation vials (6 dram) had already been prepared the previous day by first making a bulk dilution of $(x + 1) \times 0.05\text{ml}$ of an acidified saline solution of ACTH added to $(x + 1) \times 0.4\text{mls}$ of KRBG-BSA, where x was the total number of ACTH incubates, giving a concentration of 3.75 mU of ACTH/ml.

Each incubate vial then received 0.4ml from this ACTH dilution and was gassed with 95% O₂/5% CO₂ before storage at 4°C until the following day.

From final cell suspensions, two aliquots of 0.2ml were added to the duplicate ACTH incubation vials, for each adrenal pair.

0.1ml was also removed in order to carry out second Coulter counts.

A final ACTH concentration of 2.5mU/ml, for each incubate, was, intentionally, slightly high in order that experiments with slightly different cell concentrations should vary only slightly in different degrees of hyperoptimal inhibition (this occurs when the ACTH concentration rises above that required to saturate receptors), rather than display the greater divergence possible in sub-maximal response conditions.

This assumed that ACTH-mediated steroidogenesis was unimpeded by any BSA contamination which will be shown, in the next and final section, (3.3), of this chapter, to exert such an inhibitory effect.

The procedure for ACTH incubation, viability and incubation termination was identical to that described for pooled adrenal experiments.

3.3 THE INHIBITION OF STEROIDOGENESIS IN RESPONSE TO ACTH BY CONTAMINANTS OF BOVINE SERUM ALBUMIN

3.3.1 Introduction

Before its eventual identification, after approximately two-thirds of the experiments had been carried out, a factor, causing a two- to four-fold inhibition of isolated cell corticosterone production, remained enigmatic and uncontrolled. The inhibition afflicted about 50% of the uncontrolled experiments and, initially, it was thought to be due to variable cell yield. Steps were taken,

as described in Section 3.2.3, to improve yield but eventually the inhibition was observed to occur non-randomly, in runs of experiments, suggesting that the cause of inhibition was not some defect, intrinsic to the basic technique. It was more likely to be due to the intermittent introduction of some factor that exerted its undesirable effects for as long as a stock solution or enzyme batch, containing it, was in use.

Having eliminated more obvious sources of variation such as buffer preparation and ACTH solution stability, attention became focused on the dispersion enzymes, particularly collagenase, which had already been suggested as a cause of such variability in cell responsiveness (Kloppenborg et al., 1967).

The eventual deduction of the true cause was delayed by the masking of inhibition by cell count depression, by a different Coulter counter; as explained in Chapter 2, this only became obvious at later stages in experimentation. The coincidental use of new collagenase batches with new batches of BSA, ordered at the same time, also prolonged the mistaken belief that collagenase impurities were responsible.

It was only when collagenase batch number was seen to be unrelated to inhibition that the potential became obvious for BSA to contribute large concentrations of even minute impurities, by virtue of the high concentration (4%) it was used at.

Eventually, by testing different batches of BSA within the same experiment, the origin of the inhibitory influence became confirmed, as described in sub-section 3.3.2.

A retrospective study of inhibited experiments, in which response to dbcAMP remained relatively intact, is given in 3.3.3.

A superficial distinction between BSA batches, of differing purity, using immuno-electrophoresis is related to inhibitory properties in sub-section 3.3.4.

The final sub-section 3.3.5, outlines the conclusions drawn from this evidence.

3.3.2 Association of steroidogenic inhibition with the use of different batches of BSA

Two batches of BSA, previously associated with inhibited experiments, were compared with a batch that had just been found to lack any apparent inhibitory effect.

Comparisons involved the basal level, the response to 2.5mU ACTH/ml and also to 7.5mM dbcAMP.

For the total of 18 incubates, 9 CBA male adrenals were distributed, randomly, into three pools, which were dispersed, separately, using the dissociation machine.

Table 3.6 gives the results of this comparison, in which BSA's 1 and 2 had previously been associated with inhibition and, BSA 3, with normal steroidogenesis.

Table 3.6. Comparison of steroidogenic responses and the viabilities of cells dispersed and incubated using different BSA batches.

BSA Batch	Steroidogenic response (R)			Viability (%)
	Basal	ACTH	dbcAMP	
1	0.3731	0.4434	1.8577	66.02
2	0.3696	0.6159	2.4655	71.59
3	0.4091	3.1756	2.6390	68.99

Experimental notes indicate that cells, dispersed in BSA 3, appeared to resuspend more easily and form few clumps, unlike the other two BSA's.

The major result in this experiment was the confirmation, under identical experimental conditions, of the effects of different batches of BSA, suspected under differing experimental conditions.

The lowered dbcAMP response and slightly reduced viability of the cells dispersed in BSA 1 may have indicated that, in addition to an inhibitor of the ACTH response, this albumin may have contained a cytotoxic impurity.

BSA 2, however, indicated that inhibition of the ACTH response could occur with negligible disruption of the dbcAMP response. This finding, combined with the reduced cell clumping found in BSA 3, suggested the cell membrane to be the major target for interference by impurities in albumins 1 and 2.

3.3.3 Reactivity of cells to dbcAMP in experiments with inhibited ACTH responses

Before the comparison, just described, had confirmed the source of inhibition, other experiments, with reduced ACTH responses, also demonstrated quite vigorous dbcAMP responses. Table 3.7 compares the two responses and indicates whether the cells were produced using the pooled or individual adrenal pair technique.

An experiment, to be described fully in Chapter 4, compared the ACTH and dbcAMP responses of DBA, C57 and their $F_1(N)$, under controlled BSA conditions. The dbcAMP responses can be seen to be similar to their ACTH responses, in Table 3.8.

Table 3.7. Comparison of responses to ACTH and dbcAMP in experiments with inhibited ACTH responses.

Pooled/Ind.	Strain	Sex	ACTH	dbcAMP	Concentration cAMP/mM
Pooled	C57 (different line)	Male	1.86	4.88	5
*Ind.	DBA	Male	0.56	2.54	5
Pooled	(C57	Female	1.13	2.80	7.5
	(DBA	Female	0.85	2.52	7.5
*Ind.	(N	Male	1.57	3.88	7.5
	(DBA	Male	0.78	3.32	7.5

*Duplicated ACTH and dbcAMP incubations.

Table 3.8. Comparison of uninhibited ACTH with dbcAMP-induced responses.

Strain	pgms. Corticosterone Produced/Viable Cell/Hour (R)		
	dbcAMP 2.5mM	10mM	ACTH (2.5mU/ml)
DBA	1.88	2.14	1.97
N	3.49	4.12	3.53
C57	4.57	4.93	5.07

Comparison of the dbcAMP responses for each strain in inhibited (Table 3.7) and uninhibited (Table 3.8) conditions revealed that although minor differences existed, the majority of the inhibiting influence must be directed at the cell membrane. This represented more substantial evidence of the tentative conclusion reached in the previous sub-section.

3.3.4 Association of immuno-electrophoretically identified impurities with ACTH response inhibition.

In order to try and obtain some evidence of BSA impurity that could be linked to inhibitory effects, three different batches (lot numbers: 46C-7450, 65C-7533 and 65C-7532) were examined using the immuno-electrophoretic technique described in Chapter 2. The anti-serum used was rabbit anti-rind serum (bovine) in order to visualise all potential serum contaminants.

It can be seen from Plate 3.1 that this superficial examination revealed quite large differences in impurity quantity and type. The upper sample, a BSA exerting no disruptive effects on ACTH response, can be seen to be the least contaminated.

This evidence forms a suggestive link between observed impurities and observed inhibitory effects.

3.3.5 Conclusions concerning inhibitory factors.

The elimination of enzyme purity as a factor controlling the reactivity of cells to ACTH resulted in BSA being suspected as being a potential source of interfering proteins that would tend to exert

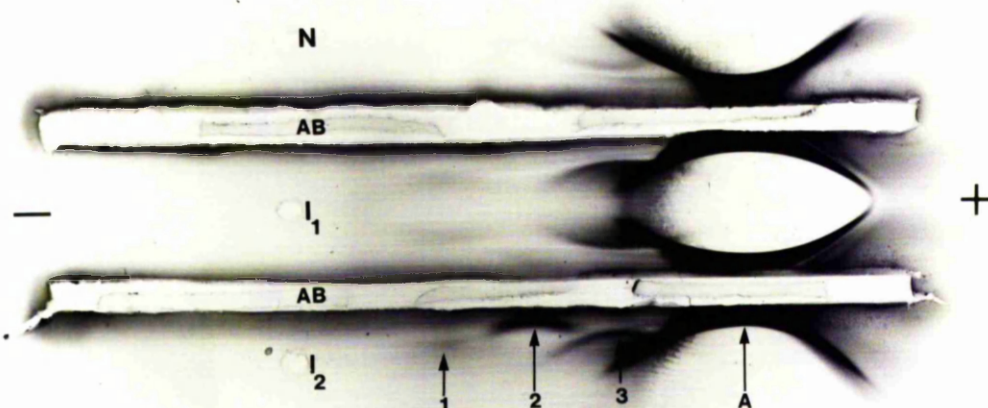


Plate 3.1 Immuno-electrophoresis of three different batches of BSA.
 I = sample wells of two BSA's known to inhibit steroidogenesis.
 N = BSA batch exerting no inhibitory effects. AB = one of anti-serum troughs.
 A = albumin immuno-precipitation crescent.
 1, 2, 3 = immuno-precipitation crescents of impurities.

a largely extracellular effect. It is possible that enzyme purity can influence responsiveness, as suggested by Kloppenborg et al., (1967), but it is also possible that these authors did not control the purity of their albumin (used at 3% concentration). It was found, by Sayers et al. (1970), in developing an improved technique for preparing rat cells for bioassay of ACTH, that although some BSA (0.5%) was essential for an optimal response, higher concentrations (2 and 3%) reduced the reactivity to ACTH from 2.79 (optimal)

to 1.73 (3% BSA) corticosterone production units. It is possible that this inhibition was of similar nature to that demonstrated in the present work.

An interfering substance could have exerted its effect by binding and so inactivating ACTH molecules but the observations, concerning greatly reduced cell clumping associated with absence of inhibition, strongly suggested the cell to be the site of interaction.

The dbcAMP response appeared to be largely unharmed by processes causing ACTH responsiveness to fall. This suggested the most likely location of inhibition-associated cellular disturbance was the cell membrane.

The immuno-electrophoretic studies revealed fairly gross differences in degrees of contamination in three batches of BSA; the most pure being the batch exerting no hindrance of the ACTH response. These impurities, being proteins, would be bound, assuming they were the effectors of interference, to remain outwith the cell membrane. As such an extracellular role had already been surmised from the dbcAMP response evidence, it was quite probable that these visualised contaminants were the true inhibitors of the ACTH response.

The attachment of a protein, to any site on the cell membrane, could have reduced the accessibility of receptors to ACTH molecules, or, more subtly, impeded the interaction of occupied receptors with adenyl cyclase.

Alternatively the inhibitor may have bound to the ACTH receptor directly, but not at the binding site for ACTH, and, by doing so,

may have altered, allosterically, the binding capabilities of the active site. The binding could also have been in direct competition with ACTH, binding at the active site.

3.4 GENERAL CONCLUSIONS

Pooled adrenal pairs could be satisfactorily dispersed using a scaled-down application of a method originally intended for dispersing rat adrenals. The cells produced by this method were found to be responsive to ACTH as well as to dbcAMP.

This method could not be miniaturised further, however, in order to disperse single adrenal pairs, without modifications to the enzymic and mechanical components of the process. Using greater concentrations of trypsin and collagenase, and an additional enzyme, hyaluronidase, combined with greater mechanical agitation, moderately high cell yields were achieved. Evidence also suggested that these alterations did not significantly alter cell responsiveness to ACTH.

These modifications were therefore incorporated in a protocol specially designed to allow upto twelve different adrenal pairs to be dispersed within the same experiment. A convenient and uniform method of applying dissociating force was also included; consisting of the mechanical alteration of air pressure within a battery of dissociating pipettes.

Manipulation of calcium concentrations was found to be ineffective in improving yield, while the use of small steel ball bearings, in order to counteract loss of enzyme permeation into adrenal tissue, caused by reduced incubate inertia, was found to raise yield. Although unadopted, it was felt that this modification would be more

likely to allow dispersion to be carried out of even smaller tissue masses than mouse adrenals, than excursions into Pasteur pipettes, especially as the limit of the usable bore size had virtually been reached.

The inhibition of the ACTH response in cells dispersed in buffer containing certain batches of BSA suggested a link between certain protein impurities, distinguished, immuno-electrophoretically, in these BSA's, and the cell membrane-situated inhibition, suggested by demonstrations of intact dbcAMP responses in the presence of ACTH response interference.

The fact that certain BSA batches were associated with ACTH response reduction was reinforced by the absence of any such inhibition (that formerly had had an incidence of about 50% of experiments) in sixteen experiments in which BSA purity was controlled.

CHAPTER 4

STRAIN DIFFERENCES IN ACTH- AND dbcAMP-
STIMULATED STEROIDOGENESIS IN POOLED ADRENAL INCUBATIONS4.1 INTRODUCTION

The results set out in this chapter, which were obtained using pooled adrenals from non-segregant mice, form two main sections.

The first of these contains a description of the ways in which corticosterone production varied for each strain, according to two major variables; ACTH (and dbcAMP) concentration and the time period of incubation. Apart from allowing knowledge to be gained concerning genotype interactions with experimental conditions, this preliminary work enabled conditions to be selected under which the phenotype, studied for the entire work, could be measured. In the absence of this basic knowledge, an ACTH concentration that produced a maximal response in one strain, but not in another, could have resulted in the spurious conclusion that the former strain had greater steroidogenic capacity. Similarly, if steroid productions, for different strains, converged with increasing length of incubation, significant differences observed on the short term might disappear over a longer period. It was thus important that subsidiary components of the response such as ACTH binding, and factors affecting the rapidity of response, should not be allowed to become limiting with respect to an appraisal of the total response.

Clarification of the phenotype in this way allowed conditions to be selected in which as little bias as possible existed for one extreme of expression over another. This required that cells were not only maximally stimulated for all strains but also that steroid

output was measured over a time scale that permitted steroidogenesis, in a state of dynamic equilibrium, to be observed for all genotypes. Such an equilibrium was envisaged as consisting of a roughly constant steroid production rate attained after the response is initially built up and ending when precursor shortages become marked. It was thought that such an equilibrium observed in vitro was most likely to represent the adrenal response in vivo, during normal stress.

Having defined the phenotype, the second major section of the chapter is devoted to comparisons drawn between strains with respect to this phenotype in order to substantiate the direct (Nandi et al., 1967; Solem, 1967; Doering et al., 1973) and indirect (Levine and Treiman, 1964; Eleftheriou and Bailey, 1972) evidence, described in more detail in Chapter 1, concerning strain differences in corticosterone production.

4.2 PHENOTYPE DEFINITION

4.2.1 ACTH dose response relationships

Responses to ACTH, and many other hormones and drugs, obey a sigmoid relationship with concentration. The mathematical and conceptual basis for such a relationship has been shown to be quite simple. The concentration of ACTH, (A), and the response it provokes, (B), have been related to the maximum response possible, (B max), and to the concentration of ACTH, (A₅₀), when B is half maximal:-

$$\frac{B}{B \text{ max.}} = \frac{A}{(A + A_{50})} \quad (\text{Sayers and Ma, 1970})$$

If this is considered as:

$$B = \frac{K_1 A}{(A + K_2)} \quad \text{where } K_1 \text{ and } K_2 \text{ are constants,}$$

B can be seen to be linearly related to $\log. (A)$ for values of A surrounding $K_2 (=A_{50})$. ACTH concentrations very different from A_{50} produce responses little different from zero or B max, depending on whether they are small or large.

In the case of ACTH, the slope of the linear portion being large, the range of concentrations between little response and virtually maximal response is relatively narrow.

Initial experiments, using pools of genetically heterogenous adrenals, were directed at establishing the approximate location of this range.

Covering a range of two orders of magnitude, the following ACTH concentrations, used in an experiment that was clearly inhibited (due to effects discussed in Chapter 3) but revealing, indicated that the linear range lay between concentrations of 0.67 and 6.7 mU.

ACTH/ml incubation medium:-

Table 4.1. Dose response to ACTH (experiment 3)

ACTH (mU/ml.)	ngms. corticosterone/incubate
0 (vehicle alone)	40.2
0.33	50.2
0.67	36.6
6.7	184
67	160

The steroid values were achieved from duplicated, chromatographically purified extracts from incubates identical in every respect except ACTH concentration.

The range was narrowed to between 0.67 and 1.33 mU/ml. In the following uninhibited experiment, steroidogenesis is expressed as pgms. corticosterone produced per cell per hour:

Table 4.2. Dose response to ACTH (experiment 6)

ACTH (mU/ml.)	pgms. corticosterone/cell/ hour (R)
0	0.021
0.33	0.021
0.67	0.816
1.33	9.443
2.67	7.791

A further experiment employing adrenals from a similar but not identical pool of mice compared the difference between impure and chromatographically purified extracts:

Table 4.3. Dose response to ACTH (experiment 7)

ACTH (mU/ml.)	pgms. corticosterone/cell/hour (R)	
	Impure	Pure
0	1.06	0.03
0.67	1.17	0.03*
1.00	1.56	0.22*
1.33	2.16	2.68*
1.67	6.00	6.01

(* only one incubate value used due to low recovery of the other)

Apart from indicating that the ACTH concentration required for maximal response was at least 1.67mU/ml., this experiment also indicated that over a large part the linear region of response, impure and chromatographically purified samples gave similar values.

The first comparison between genetically defined adrenals was carried out using those of DBA and CBA female mice. The values obtained from purified and unpurified extracts are given in table 4.4.

Table 4.4. Dose responses of DBA and CBA female cells.

ACTH(mU/ml.)	pgms. corticosterone/cell/hour (R)			
	DBA		CBA	
	Purified	Unpurified	Purified	Unpurified
0	0.06	0.24	0.07	0.36
1	0.14	0.41	0.12	0.43
1.67	0.92	1.16	1.31	1.38

Although steroidogenesis was inhibited, probably by BSA contaminants, it was possible to conclude that females from both strains possessed similar adrenocortical functions; a result expected if the sexual dimorphism observed in the expression of the adrenal lipid depletion phenotype in mature DBA's (Doering et al., 1973; Stylianopoulou and Clayton, 1976a) was to be corroborated. These results again indicated that over the abrupt linear part of the response, crude extracts gave similar values as those from extracts that had been chromatographed. It now seemed reasonable to compare dose response curves of different strains on the assumption that good approximations of their upper limits and shapes could be achieved analysing unpurified extracts.

Table 4.5 gives the results of four experiments using pooled DBA, C57 and $F_1(N)$ male adrenal cells, with duplicate incubates at each ACTH concentration.

Table 4.5. Dose responses of cells from DBA, $F_1(N)$ and C57 males.

(R = pgms. corticosterone produced/cell/hour in response to ACTH concentrations expressed)							
DBA		N		C57(A)		C57(B)	
ACTH (mU/ml.)	R	ACTH (mU/ml.)	R	ACTH (mU/ml.)	R	ACTH (mU/ml.)	R
0	0.31	0	0.36	0	0.59	0	0.76
1.17	1.21	0.67	2.87	0.33*	1.18	0.67	6.85
1.33	1.84	1.00	3.10	0.50*	1.58	1.00	7.19
1.50	2.11	1.33	3.51	0.67*	2.03	1.33	8.85
1.67	2.16	1.67	3.97	0.83*	2.37	1.67	9.49
		2.00	3.94	1.17*	3.59	2.00	9.26
				1.67*	4.61		
				2.00*	6.23		

* single incubates

These results are shown in Figure 4.1 as plots of corticosterone production versus $\log_{10}(\text{ACTH})$. Two sets of results for C57 are included to indicate that the upper limit of the linear region extended, at least, to an ACTH concentration of 2.00 mU/ml in one, unduplicated, experiment. Another, duplicated, experiment suggested that C57 was similar to DBA and N in possessing an upper limit between 1.67 and 2.00 mU/ml.

Bearing in mind the approximate nature of the dose response curves, it nonetheless appeared possible that the slope of the N curve was flatter than either parent, especially C57. The possible significance of this will be discussed, later.

Results obtained from another strain, CBA/FaCam, and its F_1 hybrid with DBA/2J, Q, are given in Table 4.6.

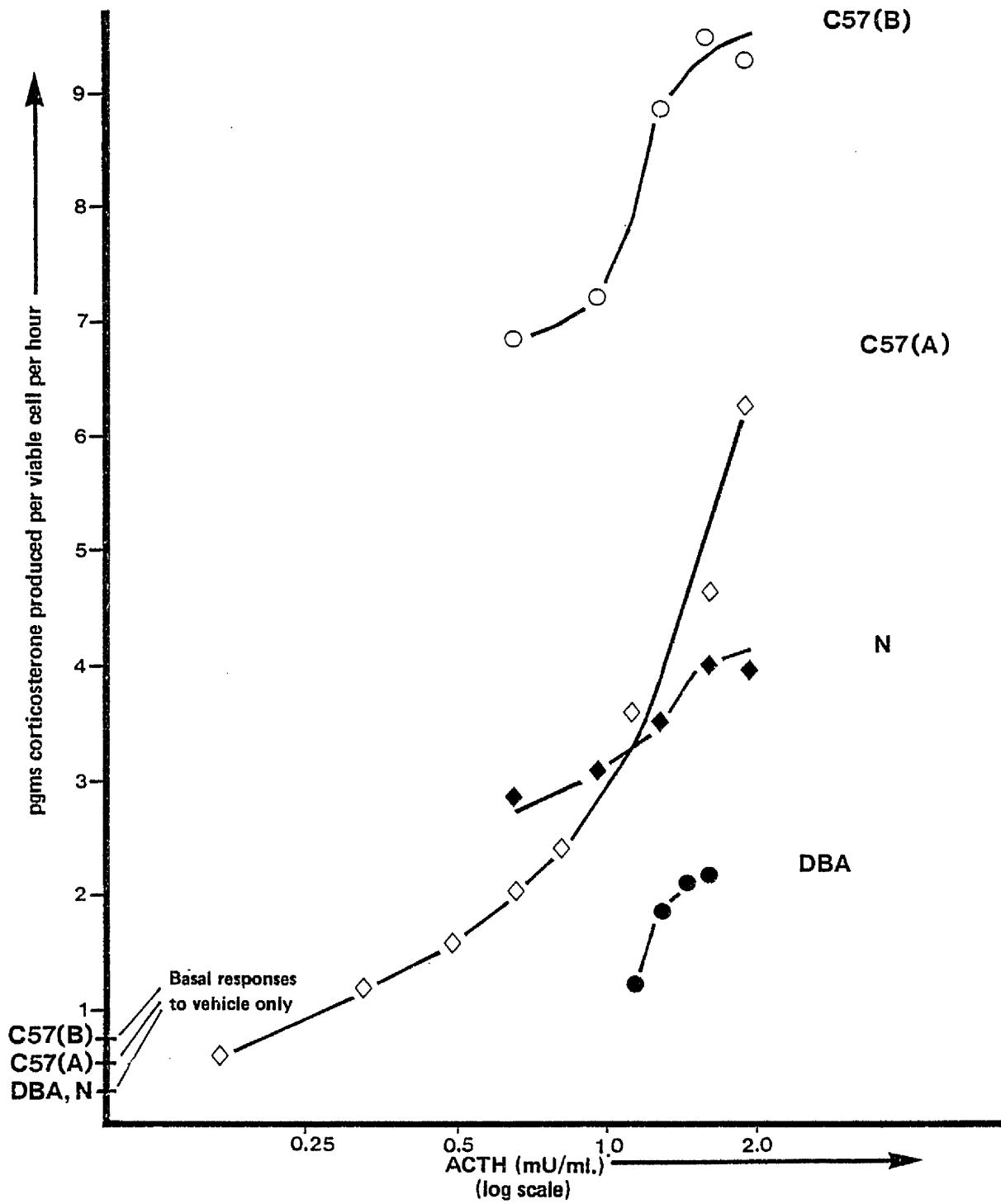
Table 4.6 Dose responses of CBA and $F_1(Q)$ male cells.

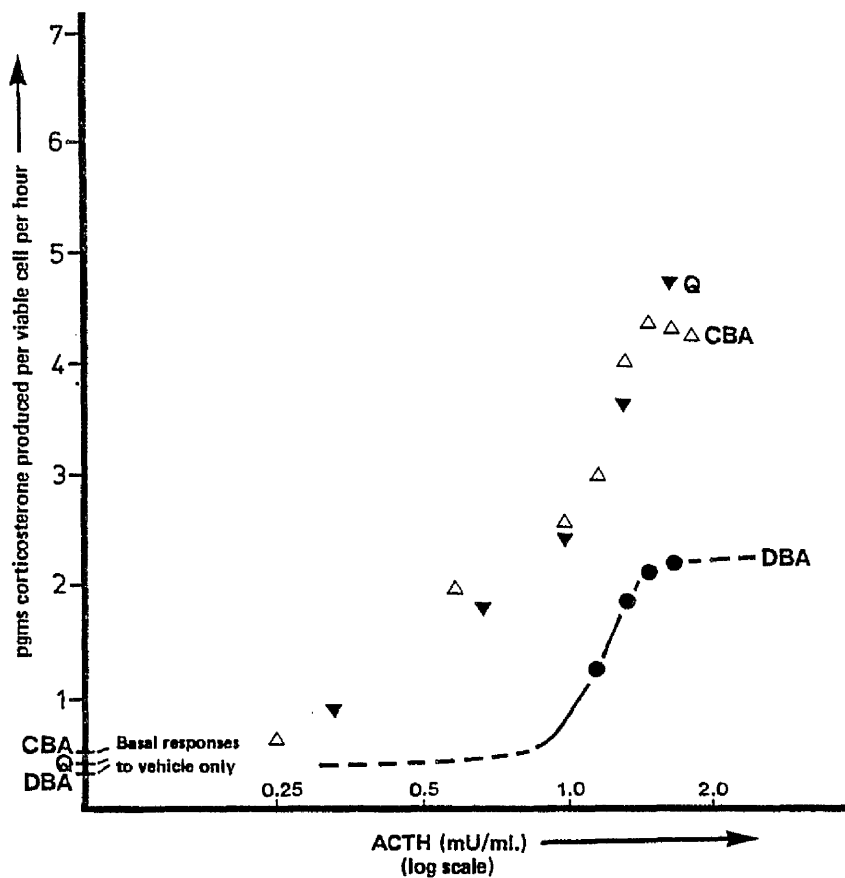
Expt:	<u>CBA</u>				<u>Q</u>			
	13		21		18		20	
	ACTH (mU/ml.)	R	ACTH (mU/ml.)	R	ACTH (mU/ml.)	R	ACTH (mU/ml.)	R
0	0.50	0	0.49		0	0.40	0	0.55
1.17	2.99	0.25	0.60		0.33	0.87	0.17*	0.61
1.33	4.08	0.59	1.97		0.67	1.79	0.33*	0.64
1.50	4.38	1.00	2.53		1.00	2.40	0.50*	0.56
1.67	4.33	1.84	4.27		1.33	3.61	0.67*	2.72
					1.67	4.72	0.83*	1.02
							1.67*	4.32

* single incubates

The dose response plots of CBA (both experiments) and Q (Experiment 18) values are compared with the earlier DBA curve, in Figure 4.2.

Figure 4.1 Dose response curves of DBA, C57 and F₁ (N) male adrenal cells to ACTH.





An ACTH concentration of approximately 1.7 mU/ml, as with C57 and DBA, appeared to result in maximal stimulation of CBA cells.

Although no crest was evident from the Q graph, the steroid production of 4.32 pgms/cell/hour, at 1.7 mU ACTH/ml, in Experiment 20 (not plotted) using single incubates, suggested that Q cells could have a virtually identical dose response to CBA.

One of the most important observations made from the dose responses to ACTH was the apparent dissimilarity in the behaviours of the F₁ hybrids, C57 x DBA (N) and CBA x DBA (Q); N cells appearing to possess quite different characteristics from those of its C57 maternal strain.

4.2.2 dbcAMP dose response relationships

The mediatory role of cyclic adenosine monophosphate (cAMP), in translating the initial signal, triggered by ACTH receptor occupancy, into increased protein phosphorylation and steroid output, has already been described in Chapter 1. Assuming any peripheral functions of cAMP were small compared with this major function, any differences between responses to cAMP and ACTH for cells of a specific strain were thought to be likely to indicate differences in the number, or binding efficiency, of ACTH receptors or other differences within the ACTH receptor:adenyl cyclase complex, resulting in altered catalysis of adenosine triphosphate to cyclic adenosine monophosphate.

Although a number of early experiments were carried out recording responses to dibutyryl cyclic adenosine monophosphate (a more permeable derivative of cAMP), the ACTH response in every case was inhibited by BSA impurities. These experiments therefore yielded no information concerning the relationship between responses to both agonists, but nonetheless, in possessing apparently intact dbcAMP responses, could be used, with the qualification that incubation conditions were not absolutely identical, to answer certain questions raised upon consideration of data obtained when BSA purity was controlled. The results from the latter experiments, possessing both ACTH and dbcAMP responses, will be considered first.

Table 4.7 gives the corticosterone productions/viable cell/hour, (R), in response to varying concentrations of dbcAMP (Sigma chemicals) and to 2.5mU/ml. ACTH; a concentration considered, on the evidence of section 4.2.1, to cause maximal stimulation in any cell type without significant depression associated with superstimulation. Also included in the table are the adrenal pool sizes

and methods of dispersion; one experiment consisted of the pooling of individually dispersed adrenals. All values were derived from one incubate except ACTH estimates and the 10 mM concentration in experiment 66.

Table 4.7. Dose responses of C57, DBA and F₁(N) male cells to dbcAMP, and their maximal responses to ACTH.

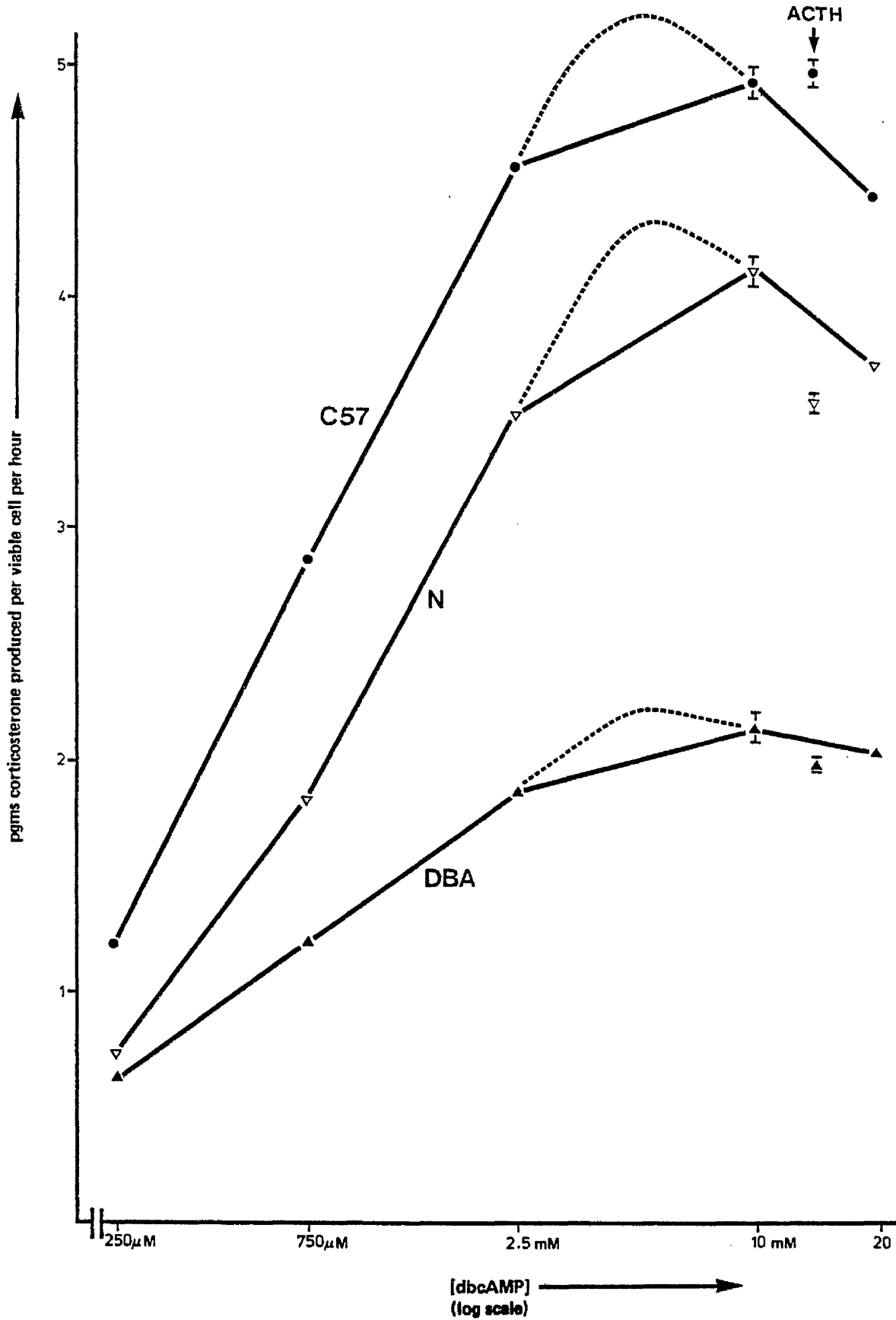
	C57			DBA		N	
Experiment:	61	64	66	64	66	61	66
Adrenal pairs:	4	4	8	4	8	4	8
Dispersion:	pool	ind.	pool	ind.	pool	pool	pool
2.5mU/ml ACTH*; (R)	3.95	2.53	4.97**	1.91	1.96**	3.04	3.54**
dbcAMP/mM							
0.25	-	0.74	1.21	0.60	0.64	-	0.77
0.275	1.22	-	-	-	-	0.97	-
0.75	-	1.24	2.86	0.76	1.21	-	1.82
2.5	-	1.49	4.57	1.23	1.88	-	3.49
2.75	3.16	-	-	-	-	2.89	-
10.0	3.36	2.99	4.93*	3.85	2.14*	3.18	4.12*
20.0	2.92	-	4.45	-	2.03	3.20	3.71

* duplicate incubates

** quadruplicate incubates.

Experiment 66 can be considered separately as it was the largest experiment in every way. It also compared all three cell lines simultaneously, and duplicated, near maximal, dbcAMP, as well as quadruplicated ACTH, responses were measured. The results of Experiment 66 are shown in Figure 4.3 in which the dotted curves indicate interpolated maxima. The means and standard errors of the

Figure 4.3 Pooled cell responses to various doses of dbcAMP and to maximal stimulation by ACTH.



quadruplicate ACTH incubates are shown to the right of the figure, as indicated. The most striking feature of the graphs is the similarity of N and C57 dbcAMP responses, in contrast to the difference, described earlier, between their ACTH responses. Furthermore, while the maximal responses to dbcAMP and ACTH appear to have been similar in C57, cells from DBA and, in particular, N, produced more corticosterone in maximal response to dbcAMP than they did to ACTH. Assuming that the response to 10mM dbcAMP was approximately maximal, t tests carried out between the duplicated dbcAMP maximal values and the ACTH quadruplicates gave the following probabilities for the mean differences:

Strain	p	
DBA	0.05	dbcAMP response greater than ACTH
N	0.01	dbcAMP response greater than ACTH
C57	N.S.	response to dbcAMP similar to that of ACTH

Although interexperimental variations in both responses were high and thus disallowed a similar statistical conclusion being reached when all three experiments (in Table 4.7) were pooled, the averages for each response follow the same pattern as those found in Experiment 66 alone. Table 4.8 gives the average dbcAMP and ACTH maximal responses for experiments 61, 64 and 66:-

Table 4.8. Maximal responses of C57, DBA and $F_1(N)$ male cells to dbcAMP and ACTH.

	Mean corticosterone produced/viable cell/hour, (\bar{R})		
	dbcAMP	ACTH	No. expts.
C57	3.76	3.82	3
DBA	2.99	1.94	2
N	3.66	3.29	3

The high average response to dbcAMP for DBA had been observed in earlier experiments in which the ACTH response had been inhibited. The following table gives the results of these experiments which also included a comparison with N cells:

Table 4.9. Responses of DBA and $F_1(N)$ male cells to dbcAMP

(Response to dbcAMP in R units)				
Expt.	<u>DBA</u>		<u>$F_1(N)$</u>	
	dbcAMP/mM	R	dbcAMP/mM	R
47	7.5	3.32*	7.5	3.88*
34	0.2	0.90		
	1.0	2.02		
	5.0	2.54		
	10	3.10		

* duplicate incubates

Cells used in experiment 47 were obtained from one individually dispersed adrenal pair, those in experiment 34 from two pairs. When these data were combined with those of the three experiments, already referred to (61, 64 and 66), the chances of the ACTH and dbcAMP means (formed from individual incubates) being compatible, as judged by t-test, were:

DBA	N
0.01	Just N.S.

It was concluded that the significant differences for both DBA and N, observed in the most definitive experiment (66), were supportable, certainly with respect to DBA cells, when all data was considered.

Only a small piece of evidence was obtained concerning any possible differences in CBA cell response to ACTH and dbcAMP. This was obtainable from the methodology experiment comparing different batches of BSA (Chapter 3, section 3.3.2) which used pools of three CBA adrenal pairs for each BSA.

The individual incubate values for each response are given in Table 4.10 for the uninhibited ACTH response and also results from a BSA batch that inhibited it;

Table 4.10. Response of CBA male cells to dbcAMP (Experiment 62)

BSA batch	Response (R units)	
	dbcAMP (7.5mM)	ACTH (2.5 mU/ml.)
Uninhibiting	2.79	3.14
	2.49	3.22
Inhibiting	2.62	0.62
	2.31	0.60

Although 7.5mM dbcAMP may not have represented a maximally stimulating concentration it seemed likely that, in view of the fact that the other strains, already described, gave approximately maximal responses at this concentration, CBA cells were apparently similar to C57 in possessing responses to ACTH that were as great, if not greater than their responses to dbcAMP, unlike DBA-like cells for which the reverse appeared to be true.

4.2.3 Strain specific variation in corticosterone production rate with length of incubation

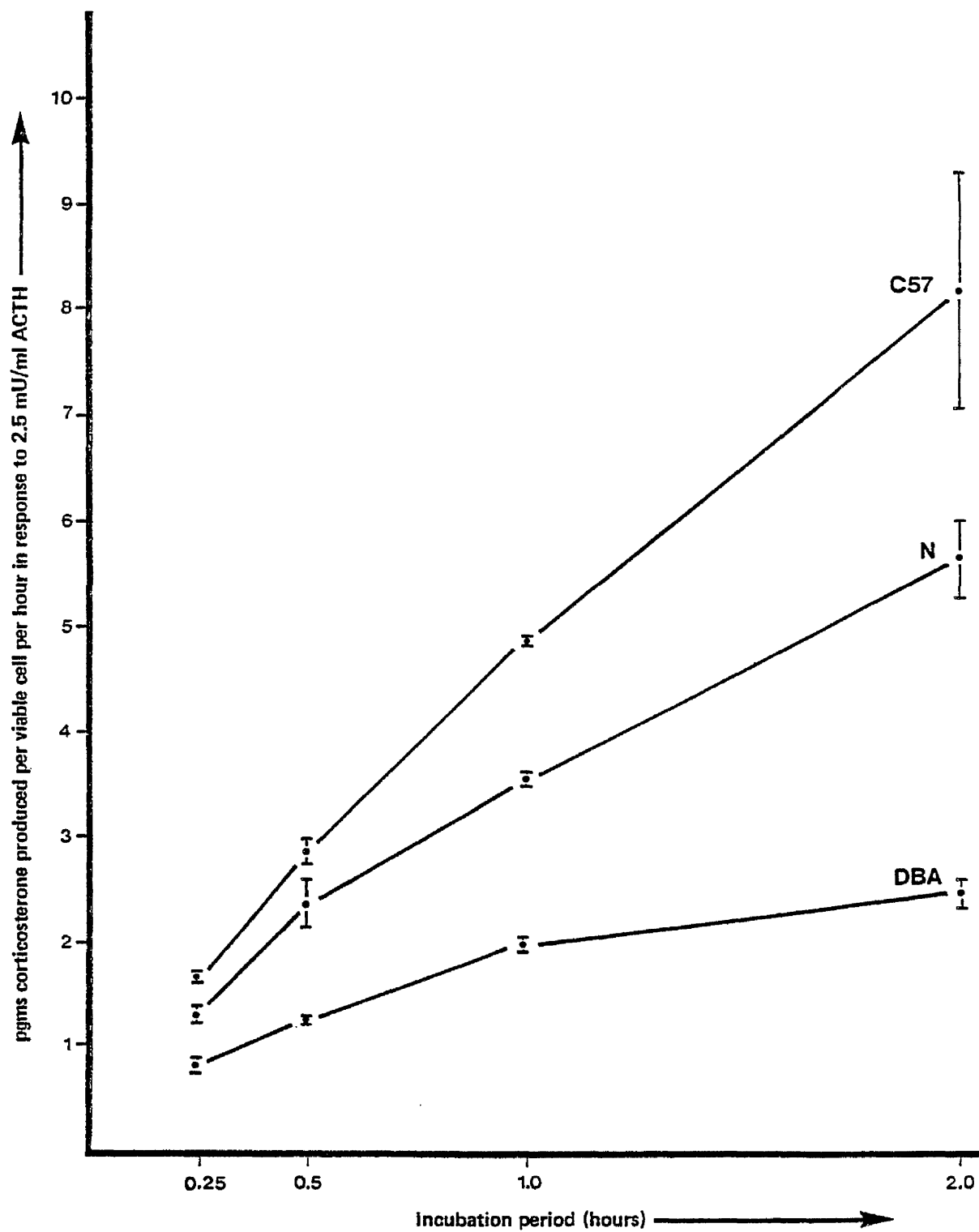
In order to select an incubation period that was likely to be moderate rather than extreme with respect to the temporal expression of sub-phenotypes such as receptor binding and extent of precursor stores, a comparison between corticosterone production rates was carried out in a major experiment utilising pools of eight DBA, C57 and N adrenal pairs. Four incubation periods were examined, with duplicate incubates for each. The results of this experiment are given in Table 4.11 and plotted in Figure 4.4; showing individual incubate values for each time period.

Table 4.11. Time course of corticosterone production by DBA, F₁(N) and C57 male cells in response to ACTH.
(ACTH concentration = 2.5 mU/ml)

Incubation period (hours)	DBA	N	C57
	Mean pgms. corticosterone produced per viable cell		
0.25	0.81	1.31	1.67
0.5	1.25	2.35	2.85
1.0	1.96	3.54	4.86
2.0	2.46	5.70	8.19

Two aspects of the phenotype can be exposed by considering the slopes of each curve between each period.

Figure 4.4 Interaction of incubation period length with strain-specific corticosterone production.



Two aspects of the phenotype can be exposed by considering the slopes of each curve between each time period:

Table 4.12. Strain specific changes in steroidogenesis with time .
Slope; (rate of corticosterone synthesis over specified time period).

Time interval (hours)	0.25-0.5	0.5-1.0	1.0-2.0
DBA	1.78	1.43	0.50
N	4.16	2.38	2.16
C57	4.73	4.02	3.33

Over the range of incubation periods studied, a fundamental facet of the general phenotype (corticosterone production/viable cell) was the marked difference between DBA and C57 in their rates of production; the lowest rate of C57 (during the second hour) being twice the highest of DBA (during the second 15 minutes). Although the absence of data before 15 minutes disallowed an unequivocal conclusion it was felt to be highly likely that such a gross difference could only be caused by sub-phenotype(s) that contributed to the general phenotype in a manner independent of time.

On the other hand, a sub-phenotype, presumed to be related to accessibility and state of repletion of cholesterol ester stores, could be shown to be time-dependent by considering changes in rates. Corticosterone production rates during the intervals (0.5-1.0 hours) and (1.0-2.0 hours) were expressed as percentages of rates during immediately preceding time intervals; (0.25-0.5 hour) and (0.5-1.0 hour), respectively:-

Table 4.13. Rates of decline of corticosterone production rates.

Corticosterone production rate as % of rate during immediately preceding time interval.		
Time interval: (hours)	(0.5-1.0)	(1.0-2.0)
DBA	80.6	34.6
N	57.2	90.8
C57	84.9	83.0

From these percentages it can be seen that whereas C57 and DBA declined in rate by roughly similar proportions during the second half hour, DBA dropped dramatically during the second hour, while C57's proportional reduction in corticosterone production remained constant. Bearing in mind the relative disparity between duplicates for N cells at half an hour, it was still thought that a significant drop in rate, to 57% of their (0.25-0.5 hour) rate, occurred during the (0.5-1.0 hour) period, compared with the slight falls to around 80% that occurred for DBA and C57 cells during the same period. It was thought that the consequent resumption by N cells, during the period (1.0-2.0 hours), of a rate of fall, similar to the 80% maintained by C57 during this final period, indicated the interaction of time-dependent and time-independent components of the general phenotype.

In summary, a possible hypothesis concerning the sub-phenotypes (possible single gene effects) comprising the general phenotype consisted of there being at least two; one being more dependent on time than the other for its expression. The former would probably consist of rate-limiting characters such as the maintainance and

utilisation of precursor pools, while the latter sub-phenotype could be thought of as basic synthetic capacity in the absence of precursor shortages. The expression of the former would also be affected by the expression of the latter. The sub-phenotypes and their effects on rate in the three strains are summarised in Table 4.14;

Table 4.14. Probable effects on corticosterone production rates of time-dependent and time-independent sub-phenotypes.

STRAIN	SUB-PHENOTYPES		EFFECTS ON PRODUCTION RATE		
	TIME DEP. (eg ester stores)	TIME INDEP. (synthetic efficiency)	Basic Rate	Curtailment	Timing of Curtailment
DBA	Low	Low	Low	Severe	Late
C57	High	High	High	Very little	Constant
N	Moderate	High?	High?	Moderate	Early

Strain specific differences in possible corticosterone degradation, by the adrenal cells, was not considered to be a major factor resulting in the severe, and moderate, curtailments of corticosterone production in DBA, and N, cells. This, and other, points will be discussed more fully in the conclusions section of this chapter.

4.2.4 Conclusions concerning phenotype definition

Having established the existence of certain components of the general phenotype which varied, strain specifically, with changes in incubation conditions, it was possible to define conditions that would be used when genetic studies were made using backcross mice. Such a definition would allow for the expression of all potential

single gene effects (hitherto observed as sub-phenotypes) also to allow them to be equally resolvable from the general phenotype.

As the ACTH and dbcAMP response evidence indicated the possibility of genetic differences at the ACTH receptor/adenyl cyclase complex, the former agonist was used in order that segregant classes with such a defect would be recovered.

An incubation period of one hour was thought to be sufficiently long to allow expression of a possible ester store effect though short enough for any differences in synthetic efficiency (steroidogenic capacity when not rate limited by precursor exhaustion) to be equally apparent.

The general phenotype measured in segregant mouse cells thus consisted of:-

The amount of corticosterone produced per viable cell maximally stimulated with ACTH over an incubation period of one hour. The proper units, pgms. corticosterone produced/viable cell/hour will be abbreviated, as mentioned before, to 'R' units.

4.3 STRAIN COMPARISONS IN ACTH-STIMULATED MAXIMAL STEROIDOGENESIS

The results concerning responses of pooled adrenal cells to ACTH were derived from four groups of experiments.

- (1) incubations that were markedly inhibited by BSA
- (2) normal values obtained from adrenals that had been dissociated manually.
- (3) normal values obtained from adrenals dissociated using a machine
- (4) a large experiment employing pools of 8 adrenal pairs (rather than the usual 5) dissociated by machine

Results in category (3) were similar to values obtained using single adrenal pairs under the same dissociation conditions (see Chapter 5) in that they were slightly depressed compared to manually-dissociated adrenal responses; a difference that was expected in view of the differences in degree of mechanical agitation (see Chapter 3). The large experiment was placed in a separate category as it appeared to contain responses midway between manually and machine-dissociated categories; an effect thought to be due to the exceptionally large adrenal pool sizes. The individual categories will be considered separately, initially, and then by correction, using correcting strains, of categories 3 and 4, a complete analysis of all uninhibited data will be presented. All t tests will be two-tailed.

4.3.1 BSA-inhibited pooled experiments

The following four experiments were markedly inhibited presumably by contaminants in bovine serum albumin used;

Table 4.15. Corticosterone production (R)

Experiment	DBA/2J	CBA/FaCam	C57BL/Tb
11		0.566	
12	0.291		
14	0.385		1.061
16	0.262		0.991
Mean :	0.313	0.566	1.026
S.D. :	0.064	-	0.049
Ratio to DBA mean:	1	1.81	3.28

The difference between DBA and C57 gave a t test probability of 0.001.

4.3.2 Uninhibited experiments using manually dissociated cells

Table 4.16. Corticosterone production (R)

Experiment	DBA	CBA	C57	$F_1(Q)$	$F_1(N)$
13	2.40	4.87			
17			5.96		
18			6.04	4.72	
19					3.94
20	2.59			4.32	
21		4.55	6.23		
Mean:	2.497	4.709	6.077	4.522	3.940
S.D.:	0.1315	0.2234	0.1355	0.2800	-
Ratio to DBA mean:	1	1.89	2.43	1.81	1.58

$$F_1(Q) = \text{CBA dam} \times \text{DBA sire}$$

$$F_1(N) = \text{C57 dam} \times \text{DBA sire}$$

t tests yielded the following probabilities, which are not expressed as percentages but as proportions of unity:

	CBA	C57	$F_1(Q)$
DBA	0.01	0.0005	0.02
	CBA	0.01	NS
		C57	0.01

4.3.3 Uninhibited experiments using cells dissociated by machine

The following R values were obtained from cells that had suffered slightly more cell membrane damage than those of sub-section 4.3.2, presumably due to the more intense mechanically disruptive effect of the dissociation machine, described in Chapter 3.

Table 4.17. Corticosterone production (R)

Experiment	DBA	CBA	C57	F ₁ (N)
25	1.58			
26	1.67		4.17	
61			3.95	3.04
62		3.18		
Mean:	1.623	3.180	4.060	3.040
S.D. :	0.0643	-	0.1591	-
Ratio to DBA mean:	1	1.96	2.50	1.87

A t test between DBA and C57 gave a probability of 0.01.

4.3.4 A large experiment employing exceptionally large pools of adrenals that were machine-dissociated

Cells derived from 8 adrenal pairs, rather than the usual 5, were used in a large experiment that examined the time-course of corticosterone production and also the responses to dbcAMP, already described. Control ACTH incubations for each part of the experiment were combined to give four replicates for each strain, pairs of which had undergone steroid extraction on separate occasions; thus yield-

ing incidental information concerning the reproducibility of the extraction procedure.

Table 4.18. Corticosterone production (R)

	DBA	C57	F ₁ (N)
Extraction A	1.91 2.01	4.82 4.90	3.61 3.47
Extraction B	2.01 1.93	5.04 5.10	3.63 3.44
Mean:	1.964	4.965	3.536
S.D.:	0.0528	0.1270	0.0963
Coeff. of variation:	2.7%	2.6%	2.7%
Ratio to DBA mean:	1	2.5	1.8

The low coefficients of variation and their constancy across strains suggest that very little experimental error was generated by the corticosterone extraction and analysis procedures.

4.3.5 Correction of machine-dissociated experiments to make them comparable to manually dispersed ones

Values from experiments described in sub-sections 4.3.3 and 4.3.4 were corrected by multiplying values by the mean for a particular correcting strain, obtained in manually dispersed incubations, and dividing by its mean within the group being corrected.

Correcting strain values were not used to provide corrected values as they would have been biased towards conformity with previous values. The correcting strain in each case was C57 and a complete table of results drawn from sub-sections 4.3.2-4.3.4 are given in Table 4.19.

Table 4.19. Corticosterone production (R)

Experiment	DBA/2J	CBA/FaCam	C57BL/Tb	F ₁ (Q)	F ₁ (N)
13	2.40	4.87			
17			5.96		
18			6.04	4.72	
19					3.94
20	2.59			4.32	
21		4.55	6.23		
25*	2.36				
26*	2.50				
61*					4.55
62*		4.75			
66**	2.40				4.33
Mean:	2.450	4.723	6.077	4.520	4.270
S.D.:	0.0938	0.1601	0.1355	0.2800	0.3089
Ratio to DBA mean:	1	1.93	2.48	1.84	1.74

* values $\times \frac{6.08}{4.06}$

** values $\times \frac{6.08}{4.97}$

t tests gave the following probabilities:

	C57	CBA	Q	N
DBA	0.0005	0.0005	0.0005	0.0005
	C57	0.0005	0.01	0.01
		CBA	NS	NS
			Q	NS

(probabilities in some cases were considerably lower than 0.0005, the minimum tabulated value).

It would thus appear that C57 adrenals produce about 2.5 times, and CBA about 2 times, as much corticosterone under defined conditions as DBA although, unlike F₁(Q), the F₁(N) appears to exhibit an inter-

mediate phenotype indicating either, only partial dominance, or at least bigenic inheritance. The effect could also arise from altered penetrance caused by a maternal effect. Further evidence of modes of inheritance, including sex chromosome linkage, could only be provided by examining genetically segregating individual adrenal pairs.

4.4 CONCLUSIONS

The results described in this chapter may appear, superficially, to have been considered in an artificial manner with, perhaps, pedantic emphasis on phenotype definition. Apart from facilitating the use of a sequence of logical steps bridging physiological phenomena with genetic ones, this emphasis on phenotype definition becomes important in drawing comparisons with previous attempts at the genetic analysis of mouse adrenal corticosterone production. Apart from indirect studies of adrenal activity mentioned in Chapter 1 (e.g. Wragg and Spiers, 1952; Eleftheriou and Bailey, 1972, among others), strain differences in adrenal corticosterone production have employed whole cell homogenates and sub-cellular fractions (Badr, 1970), organ slices of various sizes (Badr and Spickett, 1965b; Nandi et al., 1967; Solem, 1967; Badr, 1970) and whole adrenals (Doering et al., 1972). In the absence of any analyses employing isolated cells in mice, to the best of the author's knowledge, the phenotype described in the present work was unique with respect to mice and thus required careful definition.

Conclusions concerning the exact nature of the phenotype measured (corticosterone produced per viable cell per hour, in maximal response to ACTH) were described in Section 4.2.4, but the

information contained in the preceding sections, describing genotype interactions with incubation conditions, was also useful with respect to qualifying conclusions reached concerning strain-specific differences in the defined phenotype, in section 4.3.

It was apparent from the results of the latter section that the three parental strains; DBA, CBA and C57, all possessed adrenal cells that were significantly different in their corticosterone synthetic capacities. The difference between DBA and C57 was in accordance not only with previous indications that DBA was a low corticosterone synthesising strain (Blake, 1970; Doering et al., 1973) but also with the prediction that the difference was likely to be large (Shire, 1979a), as C57's, apparently, maintain their higher plasma concentrations (Levine and Treiman, 1964) in spite of greater corticosterone degradation in the liver (Lindberg et al., 1972; Shire et al., 1972).

Unlike DBA and C57, CBA strain has attracted much less attention with respect to adrenal function. A comparison of corticosterone, 11-deoxycortisol, and cortisol production by adrenal slices of CBA/FaCam and A/Cam strains, from tritiated progesterone, in the course of an eight hour incubation period, indicated that the latter strain produced significantly more corticosterone and cortisol than the former but was indistinguishable from F_1 production of these steroids. The ratios of 11-deoxycortisol : corticosterone were low for both A and F_1 , compared with CBA mice and were thought to be a reflection of more efficient 21-hydroxylation of progesterone. (Badr and Spickett, 1965b). Further work involving a third strain, SF/Cam, and the use of tissue homogenates as well as adrenal slices indicated

that SF did not produce significantly more corticosterone than CBA (although standard errors rose in proportion to the means, and logarithmic transformation of the data might have shown SF to be significantly higher). CBA and SF, being indistinguishable in the steroid ratio mentioned earlier, it was thought that the latter synthesised cortisol as well as corticosterone more efficiently. (Badr, 1970). These results, combined with the findings of similar plasma corticosterone levels in C57BL/10 and A/Jax (Levine and Treiman, 1964) and slightly higher levels in C57BL/6J compared with A/J (Chapman, 1968), both following stress, predicted that CBA mice would be likely to have a lower adrenal corticosterone synthesis than C57. The present findings have realised this; CBA cells having been found to possess only about 70% the corticosterone synthetic capacity of C57 cells.

F_1 hybrid mice, CBA x DBA $F_1(Q)$, and C57 x DBA $F_1(N)$, DBA being the paternal strain in each case, showed differences in dominance of the non-DBA phenotype. Q (CBA x DBA) was not significantly different from the CBA maternal strain, while N (C57 x DBA) was significantly different from both parental phenotypes, between which its own lay. A qualitative difference between the two F_1 's was revealed by their responses to ACTH. It appeared that unlike Q , which had a similar dose response curve to CBA, C57 and DBA, N cells exhibited a flatter response curve. In view of the lack of difference in slopes observed in the response curves, of rat isolated cells, to ACTH peptides of varying lengths and hence activities (Schwyzer et al., 1971), it seemed unlikely that the difference in the N response curve was due to altered ACTH binding. A more likely explanation of N 's different

type of response was thought to consist of a relative depletion of ACTH receptors probably due to an ontogenetic, rather than a genetic, effect, exerted in the uterine environment, a point which will be discussed further in later chapters.

Another potential cause of the difference in the N cell response to ACTH could have consisted of a relative insensitivity to cAMP, akin to that observed in a variant isolated from an adrenal tumour cell line (Schimmer et al., 1977). This was unlikely, however, in view of the observations (Section 4.2.2) that DBA and, especially, N cells appeared to have significantly greater maximal responses to dbcAMP than to ACTH, unlike C57, whose responses were indistinguishable, thus tending to support the hypothesis of relative receptor deficiency in N cells.

If a roughly equal functional depression of N and Q cells was the result of development within a non-DBA mother, the unmodified expressions of their genotypes could have consisted of virtual dominance of the C57 phenotype in N and super-dominance of the Q phenotype over CBA. (A comparison of the F_1 reciprocal hybrids of C57 and DBA will be considered in the next chapter). Q mice appeared to be more heterotic than N in terms of body weight and alertness.

The intermediary position of N between both parental phenotypes concurs with previous findings (Doering et al., 1973).

The strain specific observations on dbcAMP responsiveness, which appear to be without precedent, indicated that apart from the difference mentioned already, the concentrations giving maximal responses appeared to be very similar for DBA, N and C57 cells.

Considering the genotype interaction with length of incubation period, it was possible to propose the existence of at least one time-dependent and one time-independent factor giving rise to the differences in corticosterone production between DBA and C57 cells. A comparison between the catabolism of corticosterone, in homogenates of C57BL/ScSn and DBA adrenals, revealed that steroid reduction was higher in C57 adrenals (Shire, 1979a) thus providing evidence that the lowered steroid output in DBA was unrelated to increased degradation. This did not rule out the possibility that nascent corticosterone might have exerted strain-specific inhibitory effects on further steroidogenesis, as such negative feedback loops are thought to exist (Mulrow, 1972).

Comparison between the present time-course results and those obtained in a previous study (Doering et al., 1973) was made possible by subtracting a basal value of 10% of the 1 hour value from each value, for each strain. (This percentage was the mean for experiments in which basal values had been achieved and appeared to be quite constant across strains). A comparison is given in Table 4.20 of the ratios of corticosterone produced during the second hour over that produced in the first, for Doering's data and that obtained in this work, for which are also included the ratio of the second quarter of an hour's production over the first:-

Table 4.20. Comparison between corticosterone production by whole adrenals and cell suspensions (used in the present work)

	(Doering et al., 1973)	(The present work)	
	Second hour: first	Second hour:first	Second fifteen minutes:first
DBA/2J	0.83	0.28	0.72
C57Bl	1.38	0.68	1.09
C57xDBA F ₁ (N)	1.08	0.76	0.99

It can be seen that when comparing the second to the first hour, the phenotype measured in the present work is quite different from that measured by Doering, presumably due to the greater accessibility of cells to stimulation by ACTH. The ratios of the second to first fifteen minutes, however, appeared to resemble those of Doering more, perhaps indicating that had he extended his incubation period to four hours he might have been able to observe the very substantial drop in DBA corticosterone production observed during the second hour of the present work. Doering's corticosterone production phenotype thus incorporated a smaller time-dependent component (precursor availability) and it was thus possible that his overall conclusions underestimated the contribution of any ald genes in sustained corticosterone production.

In a comparison of plasma corticosterone levels in DBA/2, AKR and C57Bl/10 mice, following shock, the adrenal lipid depleted strains, DBA and AKR, both showed similar responses, peaking at fifteen minutes after shock. The steroid levels of C57 had still

to reach a maximum at the end of an hour (Levine and Treiman, 1964). Assuming these plasma levels reflected adrenal capacity for sustained corticosterone production, the use of isolated cells, rather than whole, or sliced, adrenals, would appear to represent, more accurately, the in vivo situation.

CHAPTER 5

ACTH-STIMULATED STEROIDOGENESIS BY
ADRENAL CELLS FROM SINGLE ANIMALS5.1 INTRODUCTION

Having ascertained that reproducible strain differences existed in corticosterone productive capacity, observed under unbiased conditions, between cells derived from pools of genetically identical adrenals, the next step was to examine cells from single animals. The reasons for this were twofold, as reflected in the two major sections of results comprising this Chapter: to obtain information concerning the spectra of responses observable among genetically identical mice (parental and F_1 lines) and also in backcross segregant animals. The variation observed in the former, being entirely governed by influences experienced during ontogeny, will be used to assess the presence of any genetically determined variation generated in the latter lines.

The results recorded are those from two distinct types of experiment: those in which varying degrees of inhibition of response were observed (for the reason described in Section 3.3 of Chapter 3) and those experiments that were uninhibited. In order to facilitate comparison and to avoid undue repetition, these two types of data will be presented together, although a distinction will be constantly maintained, both graphically and analytically, between them.

The correction of inhibited values so that they were approximately comparable with uninhibited data relied on the use of mean correction factors that were computed according to assumptions stated in the next section of this Chapter.

A brief section follows this that describes the statistical methods used; its juxtaposition to the next sections, which describe and analyse corticosterone production by cells from segregant and non-segregant genotypes, is intended to clarify, conveniently, the assumptions underlying the conclusions reached at the end of each results section.

5.2 CORRECTION OF INHIBITED VALUES

Simple correction multiples were calculated for experiments where absolute values clearly departed from normality, probably as a result of inhibition exerted by BSA contaminants.

Firstly, estimates of correction required were made for each line of animals present in experiments. These were formed by finding the ratio of the mean of all uninhibited (individual) values, for that strain, to the mean of the inhibited values under consideration.

Strain specific corrections within experiments were then averaged to give an approximate estimate of correction for each experiment.

Table 5.1 gives the strain-specific and average corrections for each experiment. From the table, it can be seen that in some cases, experimental average corrections were formed from widely divergent strain-specific corrections. A major part of this effect may be ascribed to the evidence, that will be presented shortly, of considerable ranges in normal (i.e. uninhibited) values of parental as well as segregant strains. With small samples, means of uninhibited values are likely to be only approximately equivalent to their corresponding means of normal values.

The fact that in the severely inhibited experiments (average corrections of about 4.5) DBA-specific corrections appear to be about 3.9, whereas other strain-specific corrections, in the same experiments, were generally higher, may also indicate that genetic differences may have

Table 5.1. Correction factors; consisting of the ratio of normal (mean of all uninhibited data) to inhibited steroid production, for each strain and experiment.

Expt.	No./strain	correction	average	Expt.	No./strain	correction	average
22	4	C57	4.41	4.41	3	DBA	3.38
					2	ND	3.39
	3	N	2.75	44	3	OD	2.17
30	4	CN	2.16				2.98
			2.47		2	C57	1.81
	5	DBA	3.51		4	OD	1.96
31	5	CBA	1.88	45	2	OC	1.58
			2.69				1.78
32	12	C57	2.62		2	N	4.50
			2.62	46	5	ND	5.35
	2	DBA	3.11				4.93
33	6	OC	2.14		5	N	1.76
			2.62	47	3	DBA	2.78
	2	DBA	3.91				2.27
	2	N	5.62		3	DBA	3.99
34	4	OC	3.96		3	O	4.76
			4.50	48	3	N	5.00
	2	N	3.25				4.58
37	5	ND	2.69		4	C57	4.67
			2.97	49	7	NC	4.70
	2	DBA	2.27				4.69
38	10	OC	1.95		2	DBA	3.91
			2.11	50	7	NC	5.26
	2	N	2.44				4.58
39	10	ND	2.03		2	DBA	2.12
			2.24		4	OD	1.89
	2	DBA	1.44		4	ND	1.84
	4	QD	1.07	54	2	N	3.07
42	6	OD	2.04				2.23
			1.52				
43	2	Q	1.65				
			1.65				

existed either in cells' interaction with inhibitors or in their steroid-ogenic capacities when sub-maximally stimulated.

It was possible, however, that this was merely the result of correction to a DBA normal mean that was a poor estimate of the true mean as it was formed from relatively few values.

Better estimates of correction were thought to be possible if three assumptions were made:

1. The inhibition, in all cases, was due to the presence of BSA contaminants, described in Section 3.3. (The evidence that has already been described would strongly support this assumption as none of the 17 experiments using relatively pure BSA were inhibited by any other cause.)
2. The inhibitor effects of a particular batch of BSA were repeatable and caused similar degrees of inhibition in separate experiments. (As experimental protocol was identical for all experiments it would be logical to assume that experiments involving identical levels of contaminants should undergo similar depressions of response.)
3. Experiments exhibiting similar average corrections could be grouped together on the assumption that they had been carried out using the same batch of BSA (or using different batches with similar contaminant levels.)

The third assumption was necessary as no record had been kept of BSA batch numbers during this period when collagenase impurities were thought to be responsible for cell response depression.

Table 5.2 shows the grouping of experiments with similar average corrections so that, eventually, inhibited data were corrected using only five group mean correction factors.

Table 5.2. Grouping of correction factors from experiments assumed to have been inhibited by similar amounts of BSA impurity.

Correction Group:	1		2		3		4		5	
	Expt.	C	Expt.	C	Expt.	C	Expt.	C	Expt.	C
	42	1.52	38	2.11	30	2.46	37	2.97	22	4.41
	43	1.65	39	2.24	31	2.69	44	2.98	34	4.50
	45	1.78	47	2.27	32	2.62			46	4.93
			54	2.23	33	2.62			48	4.58
									49	4.69
									50	4.58
Group Means:	1.652		2.211		2.598		2.975		4.615	

(C = average correction factor for each experiment)

The non-consecutive nature of apparent inhibition was consistent with the use of stocks of BSA; usually consisting of several batches (sufficient for 2-3 experiments) of different, although unrecorded, batch numbers that were not always used until finished.

Inhibited values were thus corrected by multiplication by the appropriate group mean correction factor. Such corrected values were not considered to be completely comparable with normal values due to the genetic considerations, mentioned earlier, and also to a slight reduction in variance, especially in severely inhibited experiments, which was thought to be largely due to the increased proportion of the approximately constant basal/background component in total inhibited responses. (It has been described in Section 3.3.2 how the inhibitors of the response to ACTH appeared to have little effect on either basal levels, or responses to dbcAMP).

In the main, use of corrected inhibited values will be shown to have resulted in little distortion, but a distinction was drawn, nonetheless, between conclusions reached using the results of uninhibited experiments and those arrived at using all data.

5.3 STATISTICAL METHODS

It will be observed, shortly, that the distributions of values for 'R' (pgms corticosterone produced per viable cell per hour, maximally stimulated with 2.5 mU ACTH/ml) are neither symmetrical nor unimodal and may, in certain cases, be discontinuous. The usual parametric statistical methods, which assume normality of distribution, were therefore inappropriate.

The non-parametric Mann-Whitney U test and Kolmogorov-Smirnov test were used in the manner described in the following sub-sections.

5.3.1 The Mann Whitney U test

The statistic U (Mann and Whitney, 1947), was found by ranking the pooled values of two samples being tested and then finding the sum of ranks for one of the samples. The following formula was used to obtain U:-

$$U = n_1 n_2 + \frac{n_1(n_1 + 1)}{2} - \text{sum of rankings.}$$

Two values for U are obtainable and using the lower one probabilities were found either directly, in the case of small samples, or by application of the formula:-

$$Z = \frac{\frac{U - \frac{n_1 \times n_2}{2}}{\left(\frac{n_1 \times n_2 \times (n_1 + n_2 + 1)}{12} \right)^{0.5}}}$$

(n_1 and n_2 are the smaller and larger sample sizes, respectively.)

This transformation to the normal Z distribution is possible when n_2 exceeds 20, whereafter the U distribution rapidly approaches normality.

Although all U test results are presented as single-tail probabilities, attention is drawn to those tests requiring a double-tailed result (double the single-tail probability) on account of the absence of any pooled incubation data to indicate any direction of difference.

The U test is used in the following sections as an indicator of difference in central tendency or location, and will be abbreviated to either M-W 1-tail or M-W 2-tail.

5.3.2 The Kolmogorov-Smirnov Two-Sample Test

This test estimates the probability of occurrence of extreme differences between two cumulative frequency distributions (Kolmogorov, 1941; Smirnov, 1948). When any absolute difference is used, the test is double-tailed and is sensitive to any difference between distributions i.e. shape as well as location.

Where the maximum difference is in a predicted direction, a single-tailed test can be used to test the alternative hypothesis that one sample is stochastically larger than the other.

The underlying assumption, of the Kolmogorov-Smirnov two-sample test (abbreviated to K-S 1-tail or K-S 2-tail), of continuity may not have been completely met in the results to be described shortly; there being good evidence of discrete groups within, particularly backcross, distributions. The test is still valid under these conditions but gives a more conservative estimate of probability of difference (Siegel, 1956).

Critical values of maximum difference (D) were obtained by a number of means.

When each sample contained at least 40 values, double-tailed estimates of D were obtained from the expression:

$$D = \text{multiple} \times \left(\frac{n_1 + n_2}{n_1 n_2} \right)^{0.5}$$

The multiple in the above formula was obtained from a table and varied according to the level of significance being examined.

Single-tailed probabilities for samples of all sizes were obtained from a transformation into the chi-squared distribution with 2 degrees of freedom:

$$\text{CHI-SQUARED} = 4 \times (\text{OBSERVED DIFFERENCE})^2 \times \frac{n_1 \times n_2}{(n_1 + n_2)}$$

Siegel, who describes the above methods, states that the chi-squared estimation gives good, although conservative, estimates of probability of difference, even when the two samples are small and of different size.

Neave (1978), however, provides single-tailed critical D values for small samples of unequal size. His tables were used when samples were less than 25 in size; good conformity was observed between this and the chi-squared method.

5.3.3 The F test

Although it assumes normality of distribution, the F test was used to test for significant differences in variances between:-

- (a) normal (uncorrected) and corrected data, within a given strain.
- (b) segregant and parental line data.

Variance estimates were formed from coefficients of variation:

$$V \text{ (coefficient of variation)} = \frac{\text{standard deviation} \times 100}{\text{mean}}$$

$$F = \frac{(V_{\text{LARGER}})^2}{(V_{\text{SMALLER}})^2}$$

It is not the intention to use this crude test to make strong assertions concerning probable differences but rather to draw guarded attention to possible differences.

With respect to its use in indicating possible differences between corrected and uncorrected values, it will only be shown within a table containing all data for a given strain when it has yielded a significant probability.

The K-S 1- and 2-tailed tests carried out on normal and corrected data within a strain, will also only be shown if they are significant.

5.4 PARENTAL AND F₁ HYBRID STRAINS

5.4.1 Results

Tables 5.3 to 5.6 give the R values (R = the defined phenotype; pgms. corticosterone produced per viable cell per hour, maximally stimulated by 2.5 mU ACTH/ml) for cells dispersed from individual adrenal pairs of the three parental strains; DBA/2J, C57BL/Tb and CBA/FaCam, and the F₁ hybrids; N(C57 dam x DBA sire), O(DBA dam x C57 sire) and Q(CBA dam x DBA sire).

Any statistical differences between normal and corrected values are given at the bottom of the tables.

Table 5.3. Parental Strain: DBA/2J

Normal values		Corrected values			
Expt.	R	Expt.	R	Expt.	R
28	1.43	31	1.65	42	2.34
	1.67		1.66		2.66
	1.65		1.58		
35	3.44	33	1.64	44	2.29
	2.27		1.56		1.75
					1.73
63	2.52	34	2.25	47	1.64
	2.63		1.40		1.84
	2.33		2.59		1.72
67	1.73	38	2.57	48	2.77
			2.74		2.29
			1.43		
				50	2.37
					2.79
				54	2.53
					2.02
		Normal	Corrected	All	
Mean:		2.183	2.073	2.102	
n:		9	25	34	
S.D:		0.637	0.469	0.511	
Coeff.Variation(%):		29.2	22.6	24.3	

Table 5.4. Parental Strains: C57BL/Tb and CBA/FaCam

Normal Values		<u>C57BL/Tb</u>		Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
24	3.76	55	2.58	33	4.09	48	4.45
	3.87		4.59		2.39		5.08
	2.69						4.40
35	2.77	57	3.73	37	2.44	49	3.85
	4.08						
36	3.86	58	4.09	44	2.64	49	3.52
	3.00		4.35		4.68		3.55
					3.39		3.61
41	4.11	60	1.55	45	3.52	49	2.75
	3.12		2.93		2.75		3.48
			2.10				
52	3.79	65	4.84				
			2.87				
			2.06				

	Normal	Corrected	All
Mean:	3.368	3.563	3.456
n:	21	17	38
S.D:	0.890	0.801	0.846
Coeff. Variation(%):	26.4	22.5	24.5

Normal Values		<u>CBA/FaCam</u>		Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
27	2.68	59	2.19	31	3.08	43	2.76
	2.50		1.78		3.65		
	1.84		2.70		3.43		
	3.87				4.11		
					3.08		

	Normal	Corrected	All
Mean:	2.508	3.350	2.897
n:	7	6	13
S.D:	0.709	0.484	0.735
Coeff. Variation(%):	28.3	14.4	25.4

$$F \text{ test } \left(\frac{V_{\text{Normal}}}{V_{\text{Corrected}}} \right)^2$$

NS

K-S 2-tail test p = 0.01

Table 5.5. F_1 Hybrids between DBA and C57 (Dams of crosses given first).N (F_1 C57 x DBA)

Normal Values				Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
36	3.57	63	3.50	30	2.86	47	3.26
41	2.76		2.65		2.05		4.75
51	2.63		1.84		2.92		3.28
			3.38		2.65		3.52
			1.86	34	1.90		2.60
	2.71		2.34				2.61
52	2.36	68	2.71		2.47		2.44
			2.74	37	2.59	48	2.60
	4.18						
	2.55				2.66		1.89
53	2.34			39	2.34	54	2.10
					2.79		
				46	2.88		

	Normal	Corrected	All
Mean:	2.757	2.721	2.737
n:	16	21	37
S.D:	0.625	0.632	0.621
Coeff. Variation(%):	22.7	23.2	22.7

O (F_1 DBA x C57)

Normal Values				Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
57	7.88		2.05		2.14		3.88
			2.08		2.49		3.55
	4.08		2.28		3.65	48	3.03
59	3.02	67	1.64		2.14		
					3.85		
	5.71			68	4.29		
	6.33						
	4.34						
	4.61						
	4.01						
63	6.12						

Cont'd

Table 5.5 (Cont'd)

	Normal	Corrected	All
Mean:	3.827	3.489	3.781
n:	19	3	22
S.D:	1.735	0.429	1.616
Coeff. variation(%):	45.3	12.3	42.7

Table 5.6. F_1 Hybrid (Q): CBA Dam x DBA Sire.

Normal Values		Corrected values	
Expt.	R	Expt.	R
	2.19		1.72
29	2.21	43	2.67

	Normal	Corrected	All
Mean:	2.201	2.198	2.199
n:	2	2	4
S.D:	0.011	0.674	0.389
Coeff. Variation(%):	0.5	30.7	17.7

(Samples too small for meaningful statistical testing.)

5.4.2 Statistical analyses

The distributions of R (pgms. corticosterone produced/viable cell/hour under maximal stimulation) can be seen (Figure 5.1) to consist, in the cases of DBA, C57 and their hybrids, of at least two classes of values, which invalidated the use of parametric statistics as stated in Section 5.3.

Although the corrected values were significantly higher than the normal ones for CBA (as judged by a K-S 2-tailed test) it was thought, in view of the small sample sizes, that it was impossible to rule out the possibility that each represented a functional class giving rise to an overall distribution that was similar in modality to the other strains. In light of the pooled adrenal evidence (Chapter 4) that indicated that CBA produce 1.5-2 times as much corticosterone as DBA cells, it seemed likely that the uninhibited individual values, which were indistinguishable from DBA values, represented the lower extremity of the CBA distribution. This argument formed the basis upon which the distribution of all CBA values was considered comparable with the distributions of all values for the other strains, none of which revealed any lack of homogeneity between normal and corrected data.

Table 5.7 contains the probabilities of the null hypothesis being upheld in single-tailed Mann-Whitney U tests and Kolmogorov-Smirnov two-sample tests.

The two major blocks of data in Table 5.7 ; normal values alone, and normal and corrected values (all), will be considered separately.

Figure 5.1 The distributions of R for individual members of parental and F₁ hybrid strains.

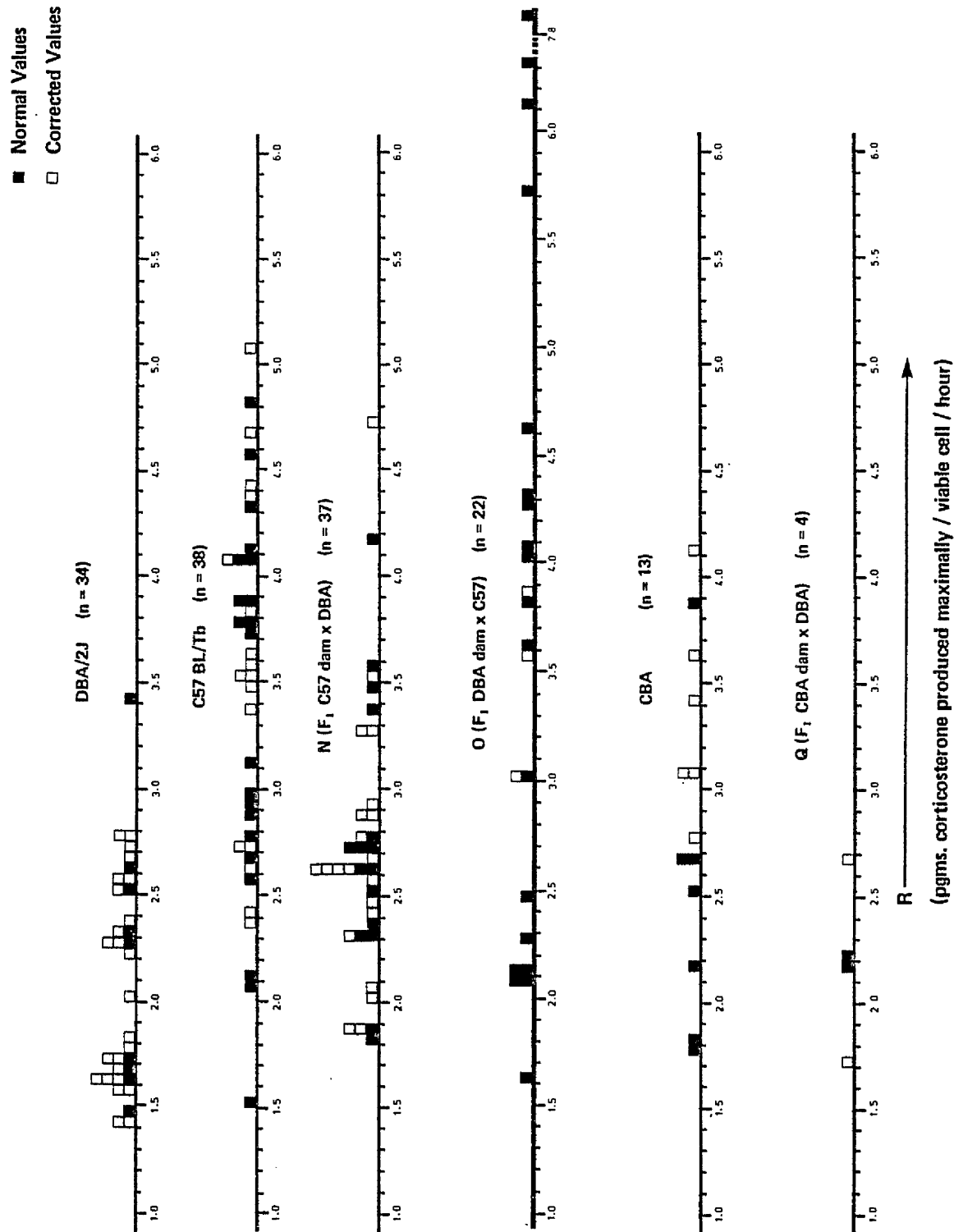


Table 5.7. Probabilities ($\times 10^2$) of parental and F_1 Mann-Whitney and Kolmogorov-Smirnov single-tailed tests.
(All probabilities expressed as %s. Stochastically higher strain underlined.)

Sample Sizes				Test	Normal Values		All Values		
Normal		All			M-W 1-tail	K-S 1-tail	M-W 1-tail	K-S 1-tail	
n ₁	n ₂	n ₁	n ₂						
9	21	34	38	DBA v. <u>C57</u>	0.1	1.0	0.003	0.1	0.1
9	7	34	13	DBA v. <u>CBA</u>	NS	NS	0.03	1.0	1.0
7	21	13	38	CBA v. <u>C57</u>	1.4	2.0	2.74	NS	NS
9	16	34	37	DBA v. <u>N</u>	1.0	2.5	0.003	0.1	0.1
9	19	34	22	DBA v. <u>Q</u>	1.0*	2.0*	0.003*	0.1*	0.1*
9	2	34	4	DBA v. <u>Q</u>	NS	NS	NS	NS	NS
21	16	38	37	<u>C57</u> v. <u>N</u>	0.8	0.5	0.003	0.1	0.1
21	19	38	22	<u>C57</u> v. <u>Q</u>	NS	NS	NS	NS	NS
21	2	38	4	<u>C57</u> v. <u>Q</u>	NS	NS	0.39	1.0	1.0
7	16	13	37	CBA v. <u>N</u>	NS	NS	NS	NS	NS
7	19	13	22	CBA v. <u>Q</u>	NS	NS	NS	NS	NS
7	2	13	4	CBA v. <u>Q</u>	NS	NS	NS	NS	NS
16	19	37	22	<u>N</u> v. <u>Q</u>	NS	1.0*	0.71*	0.1*	0.1*
16	2	37	4	<u>N</u> v. <u>Q</u>	NS	NS	3.59*	NS	NS
19	2	22	4	<u>Q</u> v. <u>Q</u>	NS	NS	3.22*	NS	5.0*

* Probability should be doubled as single-tailed test inappropriate.

$$N = F_1 (C57 \times DBA) \quad O = F_1 (DBA \times C57) \quad Q = F_1 (CBA \times DBA)$$

5.4.2.1 Normal values

In all but one case, the Mann-Whitney (M-W) and Kolmogorov-Smirnov (K-S) tests agreed; only the latter demonstrating O to be significantly higher than N. This probably reflected the fact that these two distributions differed not only in central tendency but also in compactness.

Pooled experiments (Chapter 4) have shown the R values of C57, CBA, N and Q to be significantly higher than DBA, also steroid production by C57 cells was significantly higher than that of any other line. Differences between samples of individual values thus appeared to be similar except for the lack of difference observable between DBA v. CBA, DBA v. Q and C57 v. Q.

As stated earlier, the CBA (normal) values were considered to be clustered at the low extreme of the probable population distribution, accounting for their similarity to DBA individuals. The Q results were almost certainly due to low sample size.

The significantly higher distribution of O values compared with N, as mentioned already, using K-S test, was reflected in comparisons between the two reciprocal F_1 s and C57. Whereas O was found to lack significant difference with C57, by both statistical tests, N was found to be highly significantly lower than C57.

5.4.2.2 All values

Only one test gave discordance between M-W and K-S tests; CBA v. C57. While the M-W comparison confirmed the earlier finding (Chapter 4), that C57 cells produced more corticosterone, in the conditions specified, than CBA, the absence of a significant K-S result could be interpreted as indicating that the two distributions were essentially the same, differing only in location; a characteristic to which the M-W test had greater sensitivity.

The rest of the results were similar to those using normal values only; most differences showing greatly decreased probabilities. Two differences from the normal results emerged, however; DBA v. CBA and C57 v. Q becoming significantly different when all values were used.

The difference between O and N became more marked; both M-W and K-S tests confirming the result indicated by the M-W test, only, on normal values. Single-tailed significance was found between these two F_1 's and Q, although this disappeared when the probabilities were doubled (double-tailed) on account of there being no pre-existing evidence of significant difference.

5.5 Backcrosses to DBA

5.5.1 Results

The following two tables, Table 5.8 and Table 5.9, give the values of R (pgms corticosterone produced/viable cell/hour in response to 2.5 mU/ml ACTH) for backcrosses, using the hybrid as dam, between O (F_1 DBA dam x C57) or N (F_1 C57 dam x DBA) and DBA.

The distributions of these backcross values are given in Figure 5.2 where they are compared with DBA and F_1 parental distributions.

5.5.2 Statistical analyses

Mann-Whitney U tests and Kolmogorov-Smirnov two sample tests were performed on the data for reasons stated in Section 6.4.1.

Neither F ratio test nor K-S test demonstrated any significant lack of homogeneity between normal and corrected values.

Although single-tailed probabilities are presented in Table 5.10, giving the interstrain statistical analyses, these should be doubled as no prior evidence existed that would permit a single-tailed test.

Table 5.9. Backcross ND (N dam x DBA)

Normal Values				Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
56	3.43		2.32		1.88		1.77
	2.10		0.91		2.82	44	1.89
	3.07		2.25		2.20		
			1.02		2.85		1.17
57	1.83		1.91	37	1.79		2.16
	1.82	60	1.98				2.16
					3.09		1.75
	1.43		2.55		3.09	46	1.75
58	2.98		1.42		3.28		
	1.44		1.81		1.83		3.74
		64	3.07		1.33		1.78
	2.02				2.45		3.01
59	2.06				1.60	54	1.50
	2.33				2.53		
					1.37		
				39	2.17		
Normal				Corrected		All	
Mean:		2.082		2.191		2.142	
n:		21		26		47	
S.D:		0.671		0.674		0.668	
Coeff. Variation(%):		32.2		30.8		31.2	

Figure 5.2 The distributions of R for individual members of the backcrosses (N dam x DBA) and (O dam x DBA). (DBA and F₁ distributions are shown for the sake of comparison)

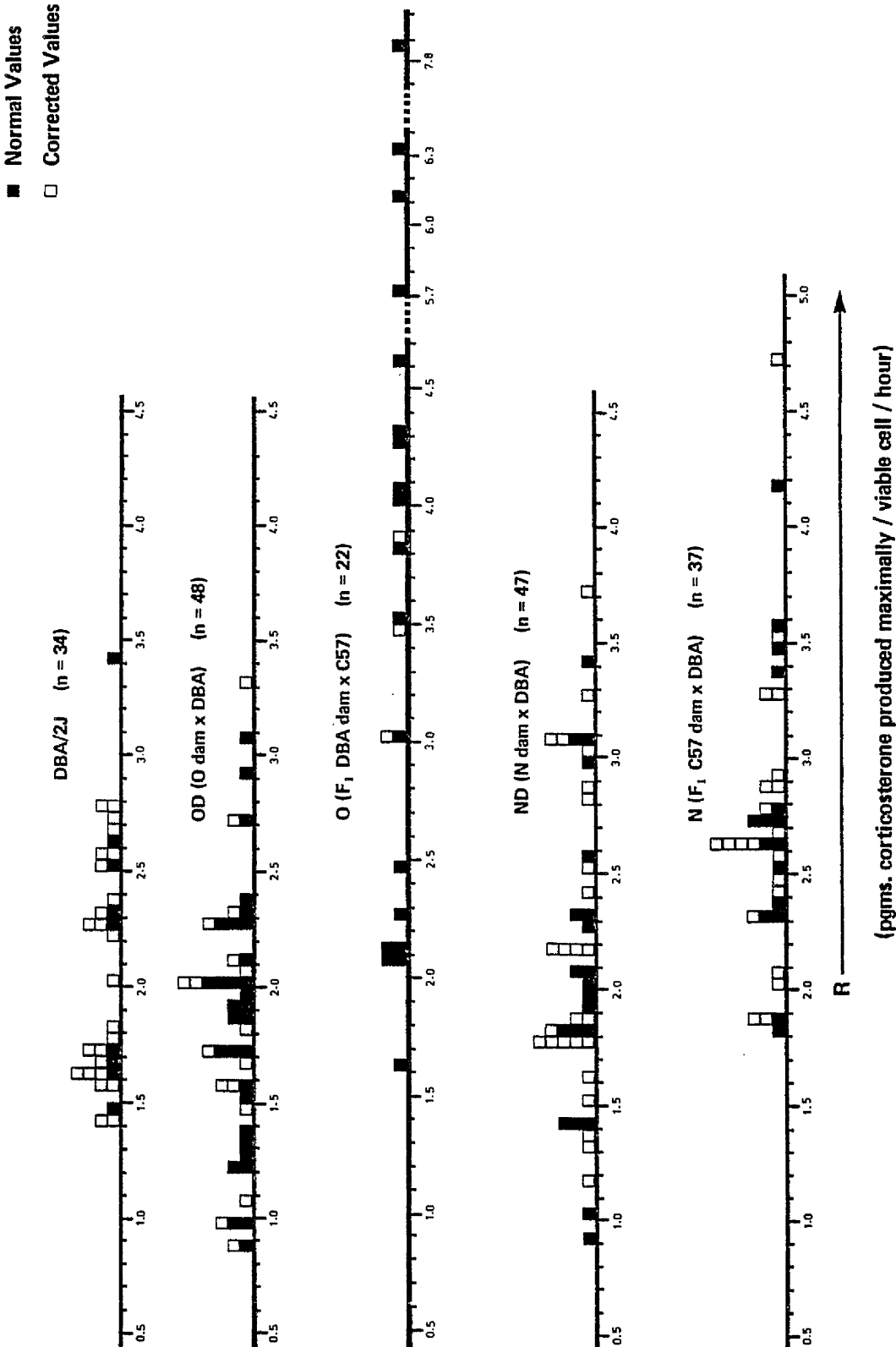


Table 5.10. Single-tailed probabilities of Mann-Whitney and Kolmogorov-Smirnov tests on OD, ND and parental strains. (All probabilities expressed as %. Stochastically higher strain underlined.)

Sample Sizes				Test	Normal Values		All Values	
Normal		All			M-W 1-tail	K-S 1-tail	M-W 1-tail	K-S 1-tail
n_1	n_2	n_1	n_2					
21	9	47	34	ND v. DBA	NS	NS	NS	NS
21	16	47	37	ND v. \underline{N}	0.15	0.10	0.003	0.10
31	9	48	34	OD v. DBA	NS	NS	3.67	NS
31	19	48	22	OD v. \underline{Q}	0.003	0.10	0.003	0.10
21	31	47	48	\underline{ND} v. OD	NS	NS	3.01	NS

OD = BC (O dam x DBA)

ND = BC (N dam x DBA)

5.5.2.1 Normal values

Both statistical tests yielded the same results. Backcross OD (O dam x DBA) and both strains were significantly different from their maternal strains, although not from their paternal strain, DBA.

5.5.2.2 All values

The double-tailed results were the same as those found for normal values.

The M-W 1-tailed tests for OD v. DBA and OD v. ND were discordant with the K-S tests in showing single-tailed significance.

It was thus concluded that OD, ND and DBA possessed similar distributions although it was possible that OD may have had a lower average than the other strains.

5.6 BACKCROSSES TO C57

5.6.1 Results

Tables 5.11, 5.12 and 5.13 give the values of 'R' (pgms. corticosterone produced/viable cell/hour, in response to 2.5 mU/ml ACTH) for backcrosses between O (F_1 DBA dam x C57), N (F_1 C57 dam x DBA) and C57. These crosses, in which the hybrid was the dam, are abbreviated to OC and NC, respectively. The reciprocal backcross ON (C57 dam x N) was also examined and values for these segregant mice are given in Table 5.13.

Although an F test, performed on the coefficients of variation of normal and corrected NC values, was significant, ($p = 0.002$), a double-tailed Kolmogorov-Smirnov two-sample test carried out on the two sets of data failed to demonstrate a significant difference. The reduced variance of the corrected values was due to the fact that basal/back-

Table 5.11. Backcross OC (O dam x C57)

Normal Values				Corrected Values			
Expt.	R	Expt.	R	Expt.	R	Expt.	R
61	4.27	65	2.79	33	5.15	38	3.20
	2.51		2.78		3.98		4.23
	3.33		3.81		4.63		4.83
	1.98		2.19		3.07		3.40
	1.97				3.20		2.09
	2.45		2.84		4.51		3.74
	3.32	67	2.07	34		45	2.61
			3.67		2.43		2.77
	4.91		2.81		4.41		1.59
	2.65		1.30		3.45		2.03
	1.70		1.54		2.20		
	2.38		2.69				2.81
65	1.94						2.79
Normal				Corrected		All	
Mean:		2.691		3.323		3.000	
n:		23		22		45	
S.D:		0.882		1.00		0.985	
Coeff. Variation(%):		32.8		30.2		32.8	

Experiment 33 values (n = 6) tested against all other values

(n = 39) gave M-W 2-tailed p = 0.005

and K-S 2-tailed p = 0.02

Table 5.13. Backcross CN (C57 dam x N)

		Normal values				Corrected values	
Expt.	R	Expt.	R	Expt.	R	Expt.	R
26	3.31		5.44		3.05		5.65
	2.96		4.97		5.18		3.45
	2.72		4.57		3.99		4.10
	2.66	35	3.64		2.85	30	3.04
	2.26				3.05		
	2.06				2.08	45	2.79
					4.29		
					2.24		
				41	2.69		
		Normal		Corrected		All	
Mean:		3.369		3.805		3.460	
n:		19		5		24	
S.D:		1.075		1.143		1.079	
Coeff.variation(%)		31.9		30.0		31.2	

ground contributions to observed corticosterone outputs, in inhibited incubations, rose in proportion to the correcting factor. This was due to the observation, already described in Chapter 4, that inhibition of response to ACTH appeared to have little effect on basal production levels. All the corrected NC values required large correction factors (approximately x 4) which diminished their variance, for the reason just described.

As the K-S 2-tail test had failed to demonstrate significant difference between normal and corrected NC values, they were combined to form an 'all' group, although the variance distortion meant that any

conclusions reached using the latter group required circumspect interpretation.

The distributions of backcross and parental strains are given in Figure 5.3.

5.6.2 Statistical analyses

Table 5.14 gives the results of both statistical analyses performed on backcross and parental values.

5.6.2.1 Normal values

The double-tailed probabilities (twice the single-tailed) were used to judge significance.

Only NC v. N and CN v. OC demonstrated divergence between the results of the two statistical tests; it seemed probable that the large difference in type of distribution was responsible for the significant K-S result in the case of the former, the largely locational difference between the averages of CN and OC giving rise to the converse.

OC appeared to be significantly different from both parental strains, whereas NC was different only from its maternal strain, N, and CN was not significantly different from either parental type.

NC was significantly different from OC but not from its reciprocal backcross, CN.

A significant Mann-Whitney result indicated that CN was different from OC in a locational respect.

5.6.2.2 All values

Significance was judged using double-tailed probabilities, and concordance between the statistical tests was observed in every test.

Figure 5.3 R values for individual members of the backcrosses (N dam x C57), (C57 dam x N) and (O dam x C57). (C57 and F₁ distributions are shown for the sake of comparison)

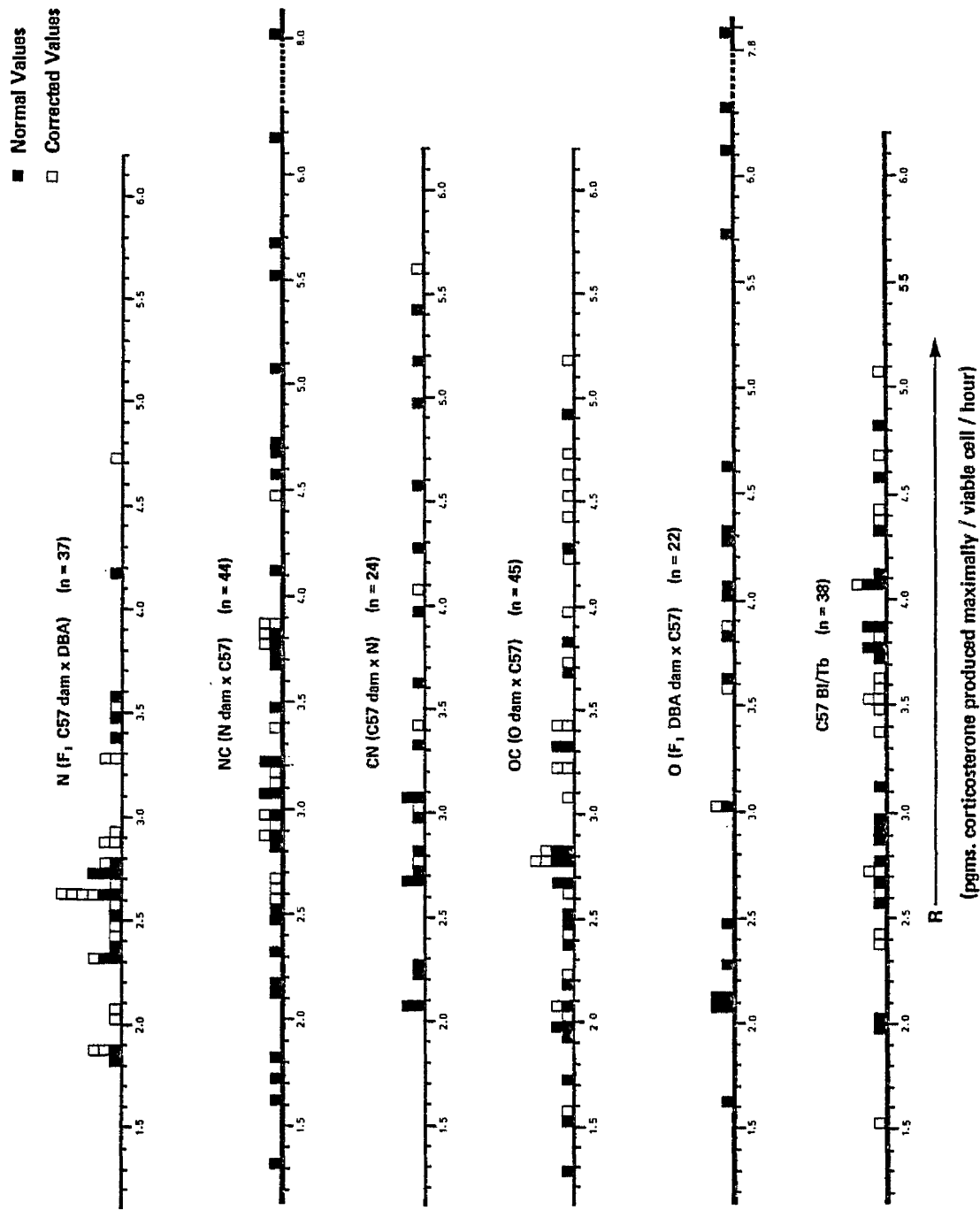


Table 5.14. Single-tailed probabilities ($\times 10^2$) of Mann-Whitney and Kolmogorov-Smirnov tests on NC, CN, OC and parental lines. (All probabilities expressed as %s. Stochastically higher strain underlined.)

Sample Sizes			Test	Normal Values			All Values	
Normal n_1	n_2	All n_1 n_2		M-W 1-tail	K-S 1-tail	M-W 1-tail	K-S 1-tail	
30	21	44	38	NS	NS	NS	NS	
30	16	44	37	4.01	2.00	0.10	0.10	
23	21	45	38	0.51	2.00	1.10(0.11)	2.00(1.00)	
23	19	45	22	1.54	2.00	3.59(1.39)	5.00(2.00)	
19	21	24	38	NS	NS	NS	NS	
19	16	24	37	5.00	NS	0.18	1.00	
30	23	44	45	1.07	2.00	3.01(0.47)	5.00(1.00)	
30	19	44	24	NS	NS	NS	NS	
19	23	24	45	1.43	NS	4.36(0.94)	NS(5.00)	

NC = BC (N dam x C57) OC = BC (0 dam x C57) CN = BC (C57 dam x N)

(Bracketted values are probabilities of tests using all OC values except those of Experiment 33).

The backcross, NC, was indistinguishable from CN, both being significantly different from their N, but not from their C57, parental types. OC, on the other hand, appeared statistically distinct from its C57 paternal, but not from its hybrid maternal strain.

Differences between NC, CN and OC followed a similar pattern to that observed with normal values alone, although only single-tailed probabilities were significant.

It was noted that Experiment 33 OC (corrected) values were significantly different (M-W 2-tailed $p = 0.005$, and K-S 2-tailed $p = 0.01$) from the rest of both corrected and normal values. The bracketted probabilities given in Table 5.14 were the result of using all OC data except Experiment 33, and are very similar to the results using normal values alone. As it was impossible to be certain that the high values of Experiment 33 were artificial (i.e., due to inaccurate correction) rather than some real difference (seasonal or due to shock), intrinsic to all data, it was impossible to discard them without introducing bias against the latter. However, the similarity of the results obtained using all values except Experiment 33, and those obtained from normal values alone, suggested that the significance observed with the normal results was not due to chance but was real.

5.7 BACKCROSSES INVOLVING CBA x DBA F_1 MICE

5.7.1 Results

Tables 5.15 and 5.16 give the values of R (pgms corticosterone produced/viable cell/hour in response to 2.5 mU/ml ACTH) for backcrosses between Q (F_1 DBA dam x C57) and the parental strains, DBA and CBA.

The distribution of 'R' for the two backcrosses and the parental types are given in Figure 5.4.

Table 5.15. Backcross QD (Q dam x DBA)

Expt.	Normal values				Corrected values	
	R	Expt.	R	Expt.	R	Expt.
25	2.19		1.74		3.13	2.47
	1.42		1.36	51	2.22	1.70
	1.75		0.84			3.37
	0.61		2.21		2.27	42
	2.55	29	1.73		1.18	3.75
	1.44			53	3.17	
	1.28					

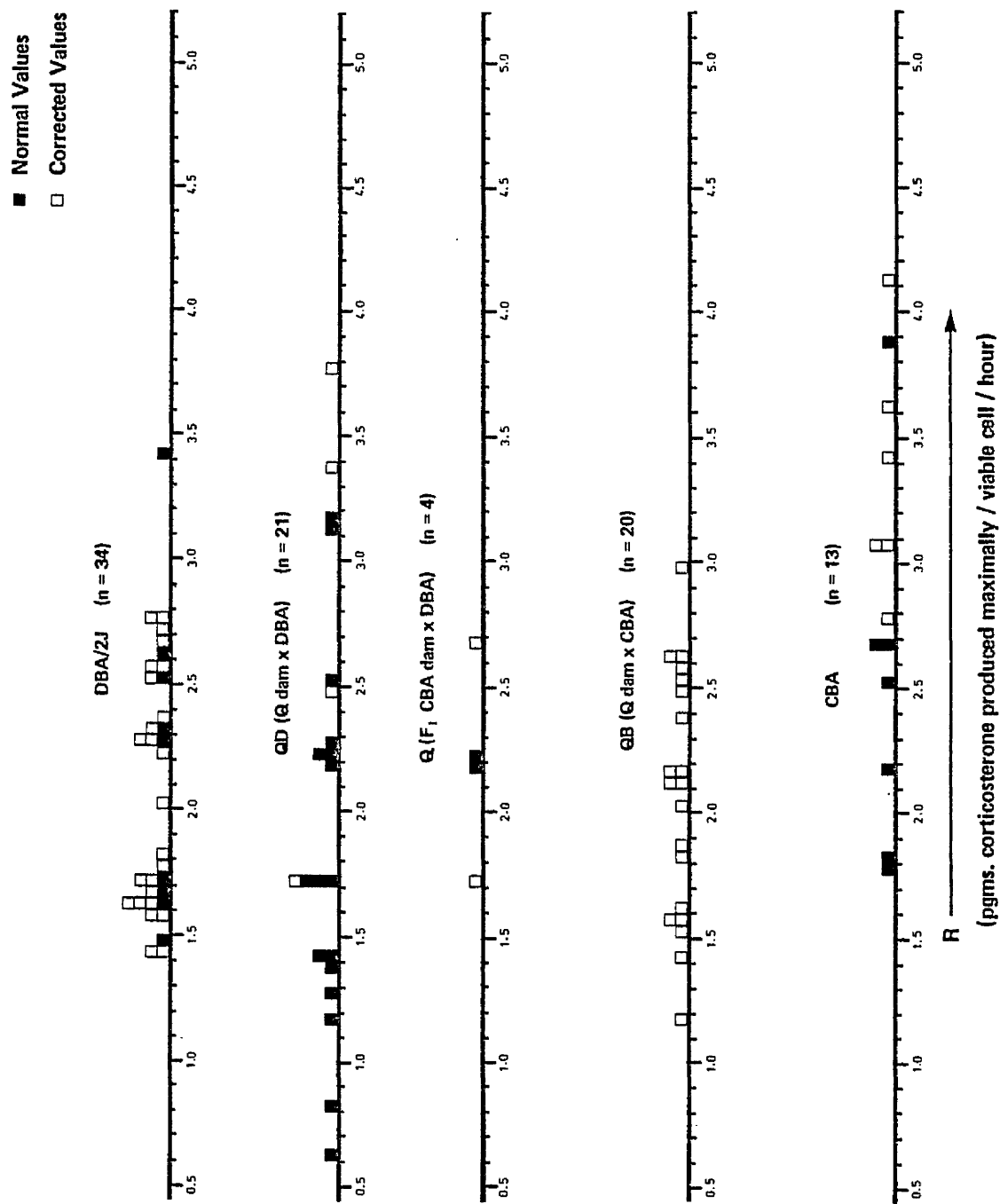
	Normal	Corrected	All
Mean:	1.829	2.823	2.018
n:	17	4	21
S.D:	0.724	0.920	0.841
Coeff.variation(%):	39.6	32.6	41.7

Table 5.16. Backcross QB (Q dam x CBA)

Corrected values					
Expt.	R	Expt.	R	Expt.	R
	2.14		2.60		2.04
	2.19		2.98		1.88
	1.65		2.48		2.58
	1.84	44	2.51		1.58
	1.56			46	2.36
	2.19		2.65		
	1.40	45	1.19		
	2.15				
43	1.54				

Mean:	2.075
n:	20
S.D:	0.485
Coeff. variation(%):	23.4

Figure 5.4 R values of individual members of the backcrosses (Q dam x DBA) and (Q dam x CBA).
(Parental and F₁ distributions are shown for the sake of comparison)



5.7.2 Statistical analyses and conclusions

All combinations of tests between the backcrosses and parentals proved lacking in significance except the comparisons of both backcrosses with CBA, which was stochastically higher in each case;

QD v. CBA (M-W 2-tail $p = 0.0024$, K-S 2-tail $p = 0.02$)

and QB v. CBA (M-W 2-tail $p = 0.001$, K-S 2-tail $p = 0.01$)

The variance of the QB distribution was almost certainly reduced as all the values had been corrected. The central tendency, however, was less likely to have been affected and it seemed probable that the general location of the distribution was similar to that of QD and DBA.

The QD data was regarded as being the most reliable source of evidence, however; consisting of many uncorrected values, it was found, using M-W and K-S 2-tailed tests, to be undistinguishable from ND and OD.

5.8 THE COMPONENTS OF INHERITANCE OF STEROID SYNTHETIC CAPACITY EXPRESSED IN SEGREGANT ADRENAL CELL TYPES

The following sections will, firstly, describe two sources of evidence of a genetic basis to the differences in the observed phenotype, R.

Differences observed between reciprocal crosses will be examined for X- or Y- linkage, after which attention will be drawn to residual, non-genetic effects.

Finally, an examination of a simple one-gene model will be described.

5.8.1 Evidence of genetic control underlying phenotypic segregation

Two major sources of information concerning the association of genetic differences with observed differences in corticosterone production; variance comparison and association with marker gene segregation, will be considered in sections 5.8.1.1 and 5.8.1.2.

5.8.1.1 Comparison of variances

In order to compare variances it was necessary to standardise them, as at least part of their observed differences between distributions appeared to be related to mean size. Coefficients of variation (V) represented standard deviations standardised to a common mean of one hundred. The following table gives the results of F tests carried out on squared coefficients of variation, for normal values, alone, and for all values:

Table 5.17. F tests on variances of normal and all values

Strain	Normal Values				n	All Values		
	n	S.D.	Mean	V ²		S.D.	Mean	V ²
OD	31	0.550	1.85	880	48	0.570	1.86	939
ND	21	0.671	2.08	1038	47	0.668	2.14	971
OC	23	0.882	2.69	1074	45	0.985	3.00	1079
NC	30	1.512	3.55	1815	44	1.288	3.47	1380
CN	19	1.075	3.37	1018	24	1.079	3.46	972
DBA	9	0.637	2.18	852	34	0.511	2.10	590
CBA	7	0.709	2.51	798	13	0.735	2.90	643
C57	21	0.890	3.37	698	38	0.846	3.46	599
N	16	0.625	2.76	514	37	0.621	2.74	514
O	19	1.735	3.83	2055	22	1.616	3.78	1817
QD	17	0.724	1.83	1568	21	0.841	2.02	1735

All Probabilities expressed as %'s

Backcross/non-segregant parent	Normal values		All values	
	F ratio	P	F ratio	P
OD/DBA	1.03	NS	1.59	5.0
ND/DBA	1.22	NS	1.65	5.0
ND/N	2.02	NS	1.89	1.0
OD/O	0.43	NS	0.52	NS
OC/C57	1.54	NS	1.80	1.0
NC/C57	2.60	2.0	2.30	0.1
CN/C57	1.46	NS	1.62	NS
OC/O	0.52	NS	0.59	NS
NC/N	3.53	2.0	2.68	0.1
CN/N	1.98	NS	1.89	5.0
QD/DBA	1.84	NS	2.94	0.5

F tests on the variance estimates, of normal values alone, revealed no significant difference with the exception of NC which had a significantly higher variance than both C57 and N. The lack of significant difference in the other tests was probably due to small sample size combined with lack of normality of the underlying distributions. Apart from the high value for O, the parental range of

V^2 values (514-852, excepting 0) did not overlap with that of backcrosses (880-1815).

When all values were used, however, nearly all backcrosses showed significantly higher variance estimates than parental strains. CN was not significantly higher than C57, probably for the reasons stated before.

Any test involving 0, for normal and all data, was not significant due to the low sample size of 0, and also to a non-genetic effect, to be described shortly.

It thus appeared likely that, as significantly greater phenotypic heterogeneity appeared in those generations with greater genetic variety, differences between parental strains were, in part at least, of chromosomal origin.

5.8.1.2 Segregation with genetic markers

Of the three coat colour, and the two electrophoretic markers, two showed significant correlation with the phenotypic segregation observed in one or more backcrosses. These relationships are described in the next two sections.

5.8.1.2.1 Isocitrate dehydrogenase (Idh-1) and malic enzyme (Mod-1)

The Idh-1 scores in relationship to corticosterone synthetic ability (R) for the backcrosses OD, ND, OC and NC are shown in Figure 5.5 for normal values, and Figure 5.6 for all data.

The electrophoretically separated isozymes were revealed by staining either, as single homozygous bands (DBA-like were slow moving, C57-like were fast-moving), or as triple banded heterozygotes.

Figure 5.5 Idh-1 segregation and steroidogenic capacity (R) using uncorrected values only.

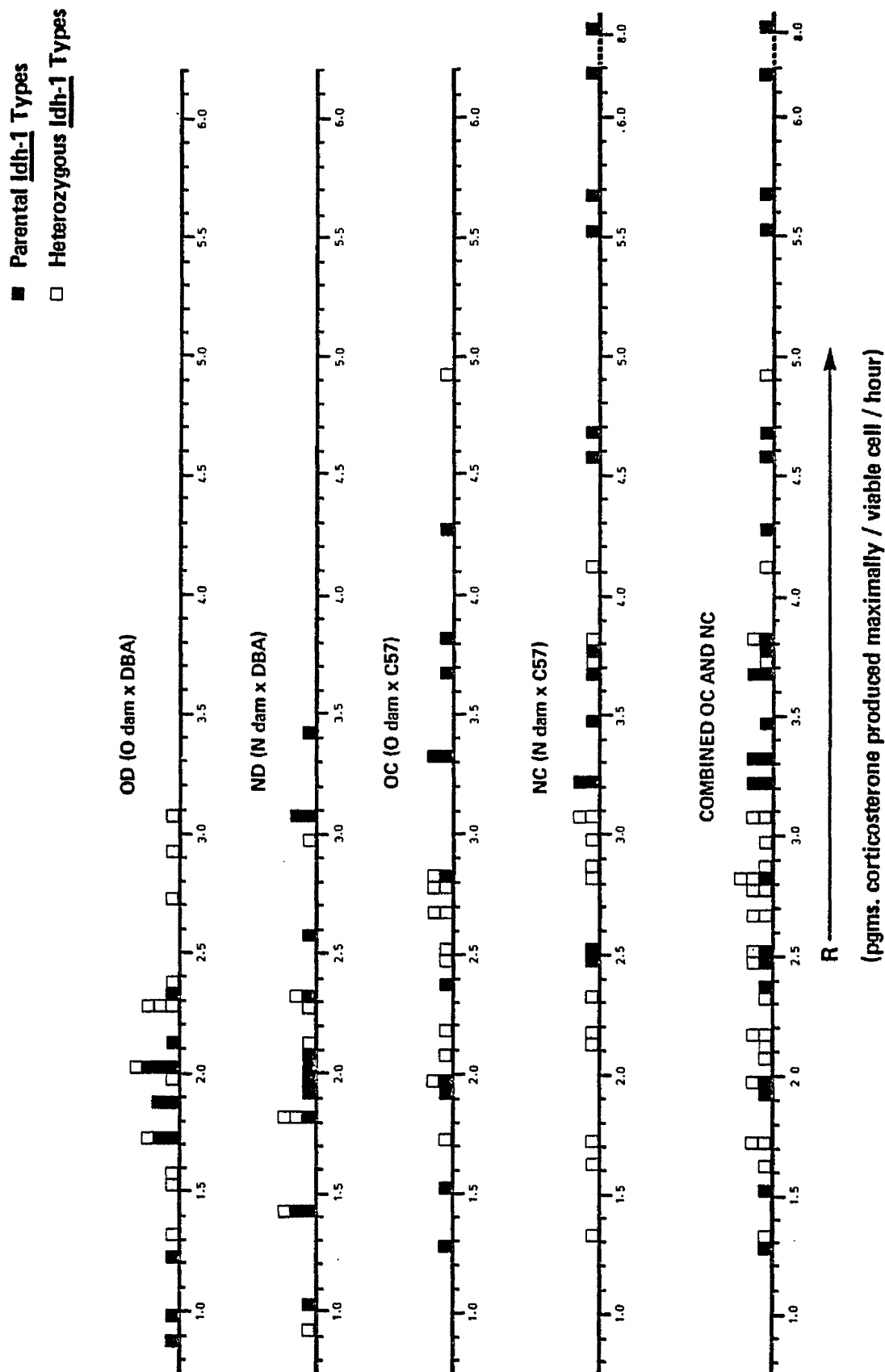
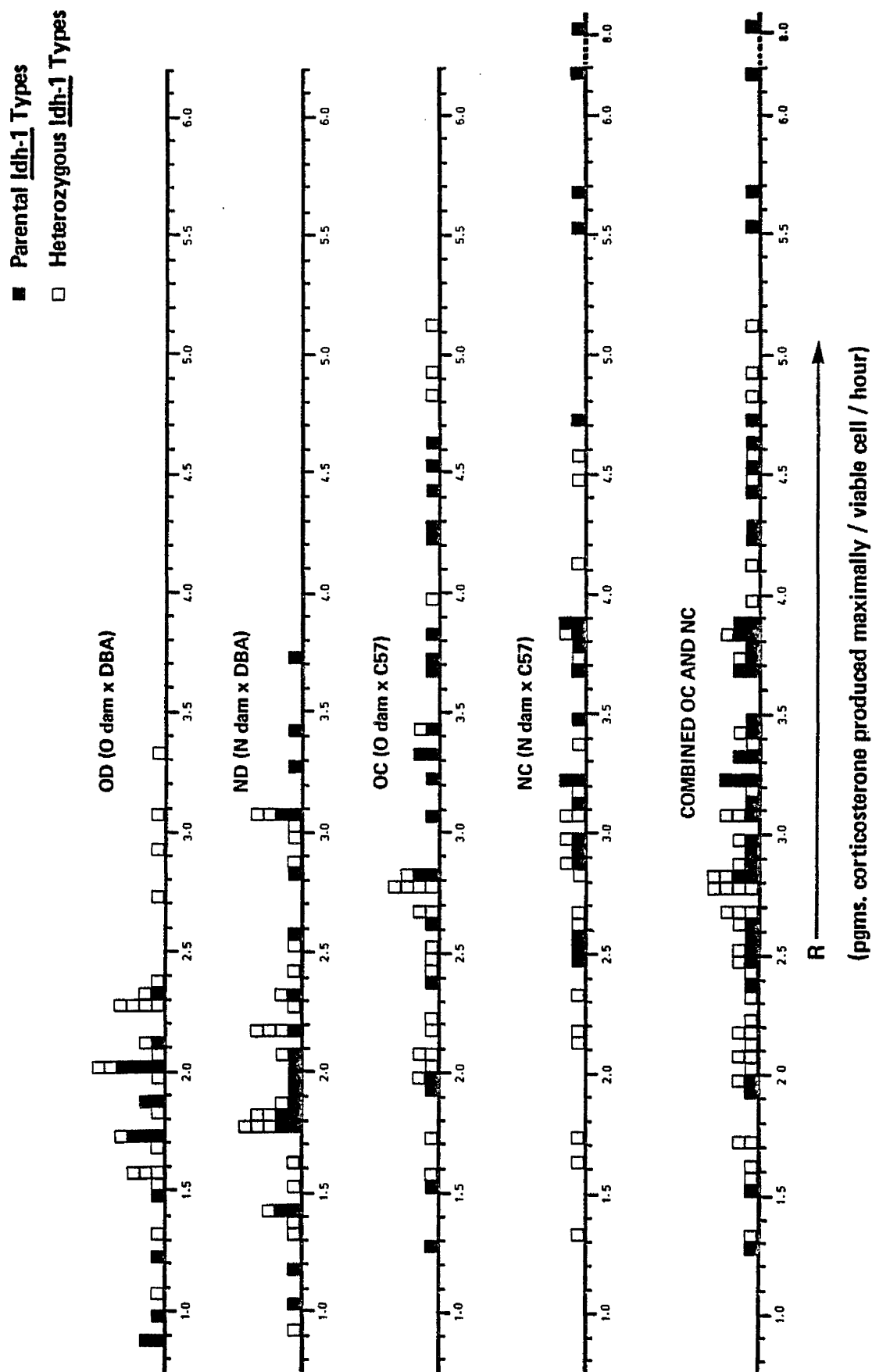


Figure 5.6 Idh-1 segregation and steroidogenic capacity (R) using corrected and uncorrected values.



When normal (uncorrected) R values, shown in Figure 5.5, were tested for significance, NC gave a probability of $p=0.02$ (M-W 2-tail) and $p=0.04$ (K-S 2-tail) of the association between high R value and slow (C57-like) Idh-1 score being due to chance alone. The other backcrosses lacked significant associations although OD gave a M-W 1-tail $p=0.057$, and K-S 1-tailed probabilities for OD and OC were between $p=0.05-0.10$. The combined OC and NC distributions, however, gave higher significances: M-W 2-tail $p=0.014$ and K-S 2-tail $p=0.02$.

Analysis of corrected, as well as uncorrected, R values resulted in significant differences being evident in OD and OC, but not ND, Idh-1 scores, as shown in the following table.

Table 5.18. Single-tailed probabilities of Mann-Whitney and Kolmogorov-Smirnov tests of differences in R associated with Idh-1 type.

All probabilities expressed as %'s				
Sample sizes (<u>Idh-1</u>)		Backcross	M-W 1-tail	K-S 1-tail
Heterozygotes	Homozygotes			
<u>24</u>	16	OD	2.68	5.0
25	22	ND	NS	NS
23	<u>21</u>	OC	NS (6.8)	1.25
20	<u>20</u>	NC	1.07	NS
43	<u>41</u>	NC + OC	0.21	0.25

(stochastically higher Idh-1 type underlined)

When probabilities have been doubled to give tests that assume no direction of difference, all backcrosses except ND can be seen to exhibit a significant difference in at least one test.

The apparent lack of significant correlation between backcross ND corticosterone production values, (R's), and Idh-1 genotype was unlikely to have been due to type II statistical error, on account of the large sample sizes. Closer examination of the difference between ND and OD showed it to consist, largely, of a difference associated with Idh-1 homozygous (DBA-like) genotypes. Thus, for OD, they were found to be associated with significantly less corticosterone synthesis than ND Idh-1 homozygotes; M-W 2-tail $p=0.026$, K-S 1- and 2-tail tests NS. Idh-1 heterozygotes from each backcross, on the other hand, possessed statistically indistinguishable R values, by both tests. This phenomenon will be discussed at a later stage in the context of a consideration of all the components of inheritance affecting R value.

It was concluded that strong evidence existed that, either the Idh-1 locus, itself, or a gene linked to it, was partially responsible for the segregation of the phenotype, R; the DBA-derived allele appearing to be associated with lower corticosterone synthesis.

A similar examination of malic enzyme type demonstrated the absence of a significant correlation between R value and the Mod-1 locus.

5.8.1.2.2 Coat colour markers; d, b and a

As dilute coat colour (d) segregated in addition to brown coat colour (b), in ND and OD backcrosses, two phenotypes, light and dark brown, were homozygous for the b allele, and the other two, black and grey, were heterozygous; B (black) being completely dominant to b (brown).

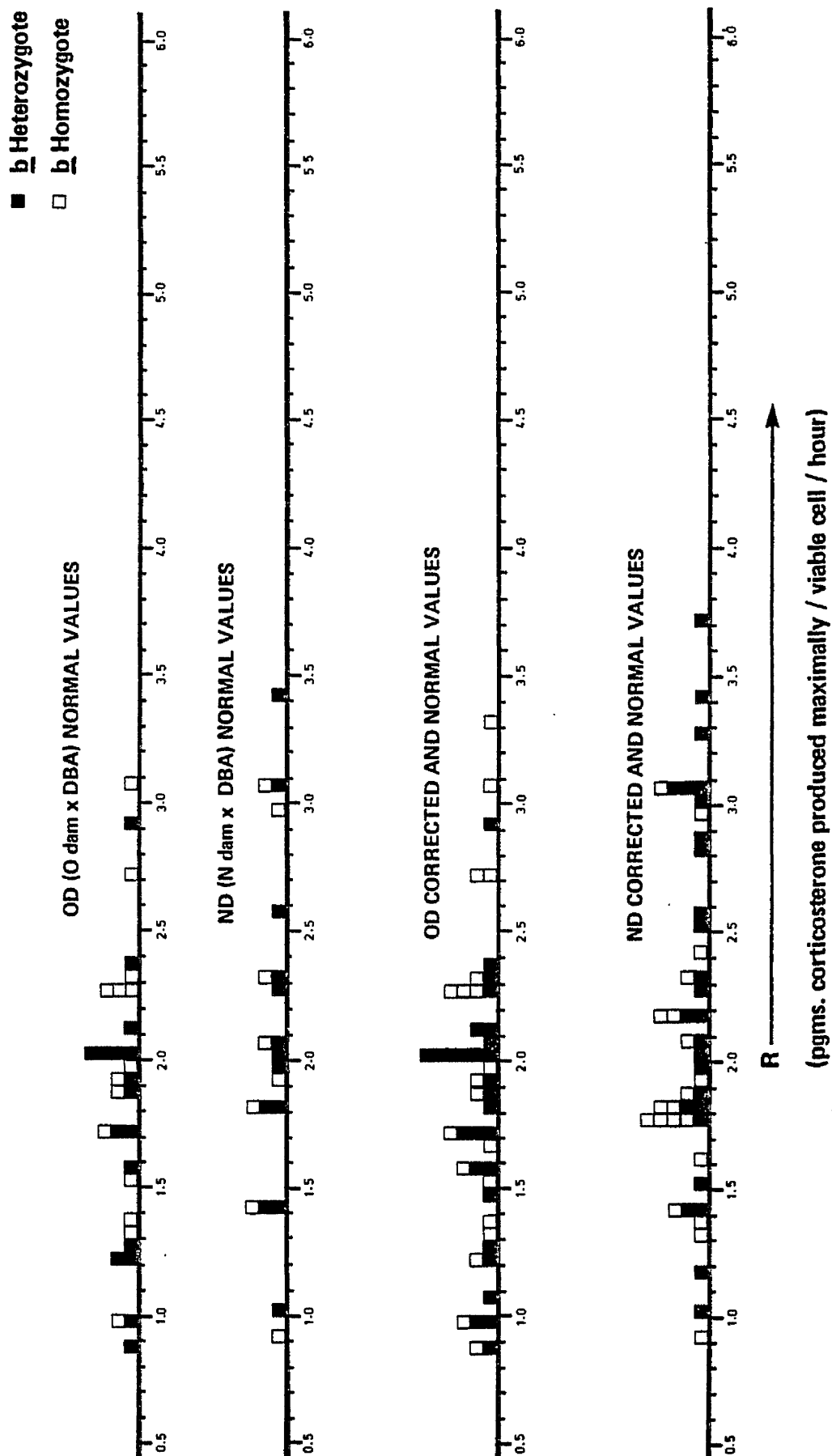
The relationship between brown coat colour and corticosterone production is shown in Figure 5.7 for normal and all values. The possibility of co-segregation, indicated by normal OD values, was strengthened by the presence of monochromatic classes, evident in the distributions of all OD and ND values. The latter distribution displayed a significant difference between homozygotes and heterozygotes for the brown gene; M-W 2-tail $p=0.047$, K-S 1- and 2-tail tests NS. No significant difference was apparent in backcross OD. Further analysis of this difference between the backcrosses showed that a highly significant difference existed between them in the R values of heterozygotes; M-W 2-tail $p=0.0088$ and K-S 2-tail $p=0.04$, whereas homozygotes from each strain had indistinguishable R distributions.

When the four possible combinations of Idh-1 and b genotype were compared between OD and ND, R values of the former were significantly lower than the latter strain only for those individuals heterozygous for b (phenotypically black and grey) and homozygous for Idh-1 (DBA-like, fast moving, isozyme); M-W 1-tail NS, K-S 1-tail $p=0.05$. A possible interpretation of this will be discussed later.

It was concluded that moderate evidence existed that the brown locus, or a linked gene, was responsible for some of the differences between DBA and C57 with respect to the phenotype, R.

Segregation at the d and a loci was analysed in an identical fashion but no significant effects were observed.

Figure 5.7 Brown coat colour (b) segregation and steroidogenic capacity (R) using corrected and uncorrected values.



5.8.2 Differences between reciprocal crosses

Two pairs of full reciprocal crosses, consisting of interchanges of maternal and paternal genotype, were bred;

1. F_1N = C57 dam x DBA sire F_1O = DBA dam x C57 sire
2. CN = C57 dam x N sire NC = N dam x C57 sire

In addition two pairs of partial reciprocal crosses were made using reciprocal F_1 dams;

1. OC = O dam x C57 sire NC = N dam x C57 sire
2. OD = O dam x DBA sire ND = N dam x DBA sire

The following table gives statistical details of these crosses and also the probabilities of either M-W or K-S 2-tail tests on paired reciprocal strains;

Table 5.19. Means, standard deviations and coefficients of variation (V) for reciprocal crosses.

All probabilities expressed as %'s										
Normal values						All values				
Cross	n	mean	S.D.	V	p	n	mean	S.D.	V	p
N	16	2.757	0.6251	22.7		37	2.737	0.6207	22.7	
O	19	3.827	1.735	45.3	2.0	22	3.781	1.6161	42.7	0.2
ND	21	2.082	0.6708	32.2		47	2.142	0.6675	31.2	
OD	31	1.854	0.5501	29.7	NS	48	1.859	0.5697	30.6	3.01*
NC	30	3.549	1.5121	42.6		44	3.468	1.2881	37.1	
OC	23	2.691	0.8817	32.8	2.0	45	3.000	0.9852	32.8	3.01*
CN	19	3.369	1.0748	31.9	v.NC NS v.OC 2.0	24	3.460	1.0787	31.2	v.NC NS v.OC 4.36*
Difference as % joint mean										
Normal					All					
N/O					32.5					
OD/ND					11.6					
OC/NC					27.5					

* single-tailed probability.

The possible causes of such differences between reciprocal crosses will be discussed in the following two sections.

5.8.2.1 X-chromosome linkage

As the backcross CN had a homozygous dam (C57), any segregant classes, of phenotype R, related to a sex-linked gene would be expected to be absent.

Comparisons between CN and NC, using normal and all values, revealed no significant difference between them, using M-W and K-S 1- and 2-tail tests.

Variance estimates (from the coefficient of variation, as described in section 5.8.1.1) for the two strains were found, by F test, to be statistically similar.

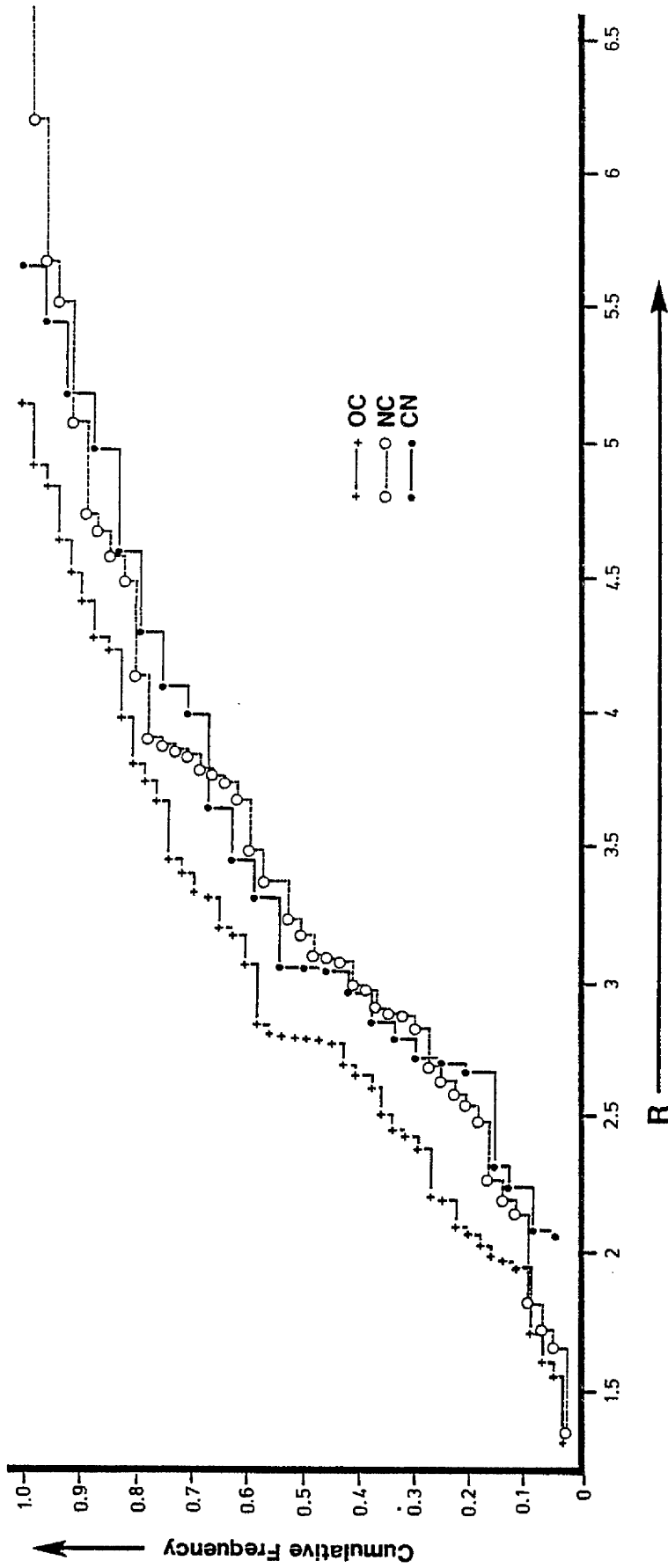
Figure 5.8 shows the cumulative frequency distributions for all CN and NC values which can be seen to run very close to each other. (The OC distribution is included for reference).

This evidence suggests that, although the sample size of CN was relatively small ($n = 24$), it was probable that the NC distribution was not significantly more varied and therefore did not contain any extra phenotypic class generated by a sex-linked gene.

5.8.2.2 Differences of apparently non-genetic origin

From Table 5.19 it can be seen that when differences in mean between paired backcrosses are expressed as a percentage of the joint mean, they were identical (14.2%) when all values were used, and this was approximately half that observed for the full F_1 reciprocals: N and O.

Figure 5.8 Cumulative frequency distributions of NC, CN and OC (all data).



As the Y-, as well as the X-, chromosome of backcross CN was different from NC, the statistical similarity of these strains indicated the probable absence of any major Y-linked difference. In addition, male chromosome linkage could not have explained the differences between half reciprocal backcross pairs (e.g. ND and OD), as each pair had the same paternal strain.

The fact that backcrosses to both parental lines were different in the same direction, depending on which F_1 reciprocal had been the dam, suggested that these non-genetic differences were probably the result of a maternal effect. This influence, whether pre- or post-natal, appeared to be exerted in a reciprocating manner, with respect to the phenotype, R; the females of strains with males with high corticosterone output appearing to produce male progeny with relatively reduced R values, while females from strains with low-producing males gave offspring with raised adrenal function. The possible mechanisms and relative importance of this developmental effect will be discussed in the conclusion to this chapter.

5.8.3 Evidence that only a few gene differences differentiate the DBA and C57 R-phenotypes

The distributions of R (pgms. corticosterone produced by one viable cell during one hour's maximal response to ACTH) of backcross individuals have already indicated, by the recovery of appreciable numbers of parental phenotypes, that the gene differences contributing to such segregation must be few.

The first of the following sections describes the construction of smoother frequency distributions by differentiation of cumulative frequency functions.

The greater resolution of phenotypic classes provided by such frequency distributions are then used in subsequent sections which test the observations against a simple one-gene model.

The model is first applied to original values and then, having described a rational, to pooled reciprocal crosses corrected in such a way as to minimise differences of maternal origin.

5.8.3.1 Differentiation of cumulative frequency distributions

With relatively small sample sizes, the overall shape of a frequency distribution may be difficult to discern if histogram class intervals are too small. In some cases, however, selection of larger class intervals results in information loss. One way of obtaining greater clarity is to use the slope of the cumulative frequency distribution as the ordinate of a frequency distribution (Stewart, 1969). A formula, obtained by fitting a quadratic equation to seven consecutive points by least-squares, allows a slope estimate at the mid-point to be calculated:

$$\text{slope at } X_0 = \frac{28}{3x_3 + 2x_2 + x_1 - x_{-1} - 2x_{-2} - 3x_{-3}}$$

where x_{-3} , x_{-2} , x_{-1} , x_0 , x_1 , x_2 and x_3 are seven consecutive values.

5.8.3.2 Test for a single gene difference using original values

In order to test for a single gene difference, composite distributions (of equal size to the backcross under test) were constructed, consisting of 50% parental: 50% F_1 values, selected randomly.

The following table gives the Kolmogorov-Smirnov double-tailed (K-S 2-tailed) probabilities associated with comparisons between observed and composite values:-

Table 5.20. Probabilities of backcross distributions fulfilling a single gene model.

Backcross	All probabilities expressed as %'s				All Values	
	Composite Distribution	Normal Values	Sample K-S		Sample K-S	2-tail
	50% parental: 50% F ₁	size	2-tail		size	2-tail
ND	DBA	N	18	NS	47	1.0
OD	DBA	O	18	NS	44	0.1
NC	C57	N	30	NS	44	5.0
OC	C57	O	23	2.5	44	1.0

A one-gene difference hypothesis appeared to be upheld by three out of four backcrosses when normal values alone were considered. The sample sizes were small, however, and probabilities were near 10%.

When corrected values were used, all backcross distributions appeared to depart significantly from those theoretically associated with a single gene difference.

Backcrosses with N dams appeared to be less significantly different, in this respect, than those with O dams.

Figures 5.9 and 5.10 show the frequency distributions, formed by the method given in section 5.8.3.1, of the four backcrosses, together with those of appropriate composites, using normal and corrected values. (Phenotypic classes at each end of distributions

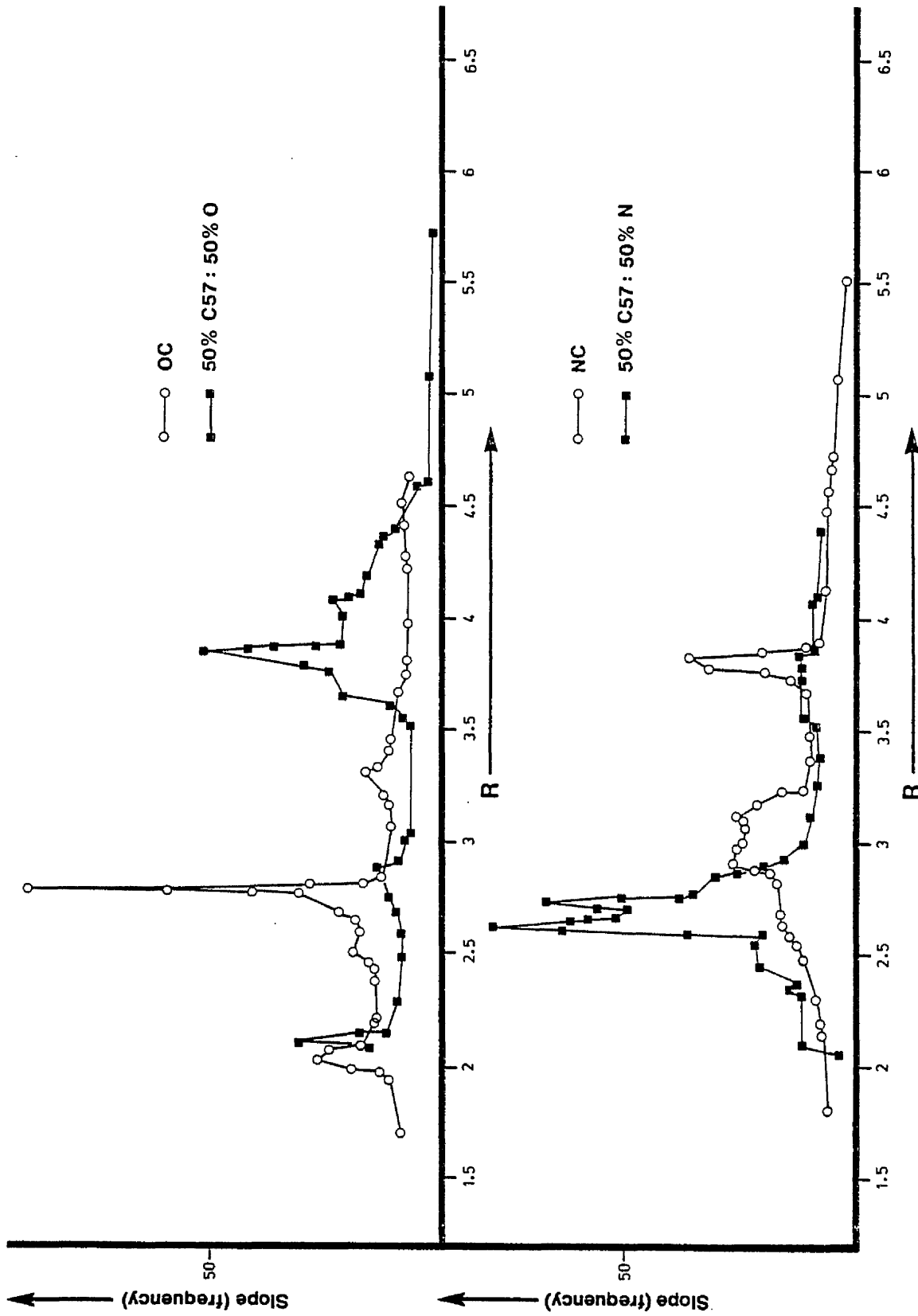
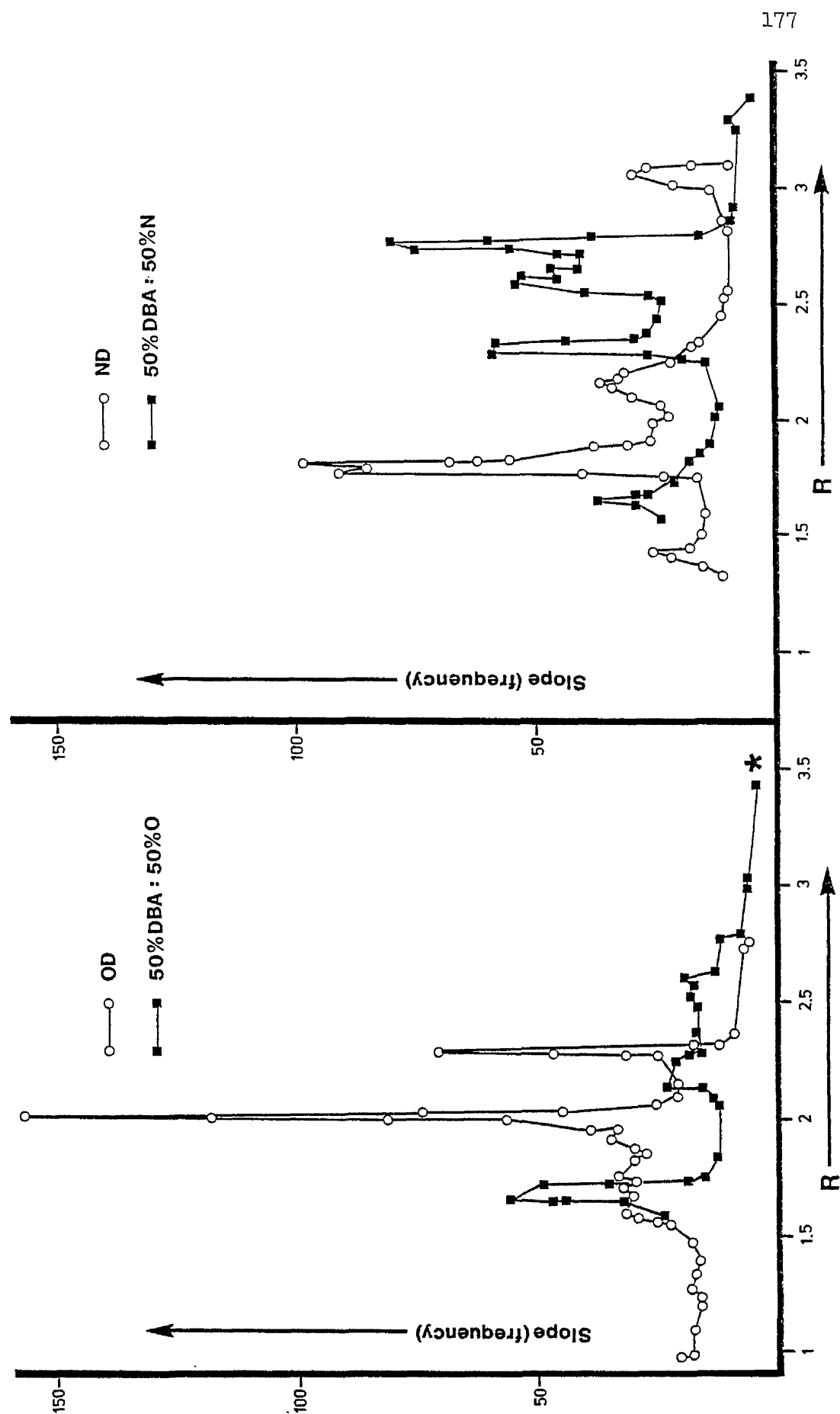
Figure 5.9 Backcrosses to C57; OC and NC with one-gene composite distributions; 50% C57 : 50% F₁.

Figure 5.10 Backcrosses to DBA; OD and ND with one-gene composite distributions; 50% DBA : 50% F₁.



contain three more values for which slope estimates could not be formed by the formula. The upper end of the OD composite distribution, marked with an asterisk, has been truncated for space reasons; there being 13 more values that form a small peak of slope = 9.05 at $R=3.846$).

In every case locational differences between observed and theoretical distributions are evident. In some cases the presence of extra classes is suggested, especially by backcross ND which appears to possess four equal classes, two of which lie outside either parental class.

In order to conclude that there were probably two gene differences, rather than one, it was important to attempt to minimise differences of presumed maternal origin and re-test values, corrected to this end, against a one-locus model.

5.8.3.3 Reduction of maternal effect

In order to minimise non-genetic (presumed maternal) differences between reciprocal crosses, their corticosterone values were corrected in the following way. The average of the means of the two reciprocals was assumed to represent, approximately, the mean steroid production value that would be obtained in the absence of maternal stimulation or repression. Values in reciprocal crosses were thus multiplied by the factor:
$$\frac{(\text{mean}_1 + \text{mean}_2)}{2 \times (\text{mean}_1 \text{ or } \text{mean}_2)}$$

where mean_1 and mean_2 were the means of each reciprocal.

Such an adjustment could only be approximate as it assumed the repression exerted by the dam in one reciprocal line was equal

and opposite to the stimulatory effect found in the other. It also assumed that segregant classes within crosses were all equally susceptible to the maternal effect. Although it was unlikely that the maternal effect was restricted to one dam and not the other, it was probable that it acted differentially on segregant classes.

5.8.3.4 Test for a single gene difference using values adjusted to minimise maternal effects.

Having applied the adjustment factor, just described, to pairs of reciprocal crosses, pooled backcrosses, consisting of 50%:50% for each reciprocal, were tested against single gene model distributions, comprising 50% DBA or C57 : 25% adjusted O : 25% adjusted N.

Using normal values only, K-S 2-tail tests indicated no significant difference between observation and model, although the backcross to DBA had a probability less than 0.1.

Comparisons incorporating all values (Figures 5.11 and 5.12), however, revealed a highly significant difference between the pooled backcross 50% ND : 50% OD and its one-gene model; K-S 2-tail $p = 0.005$. No significant difference was found between the pooled backcross to C57 and its one-gene expectation. This may have resulted from inadequate sample size ($n=76$) but, in presenting so contrasting a result to that of the other backcross, it signified the probable involvement of two gene loci; one of which may have possessed a highly dominant C57-derived allele that was indistinguishable in heterozygous or C57-homozygous forms, due to both the very specific nature of the phenotype (section 4.2) and the sensitivity with which it was measured.

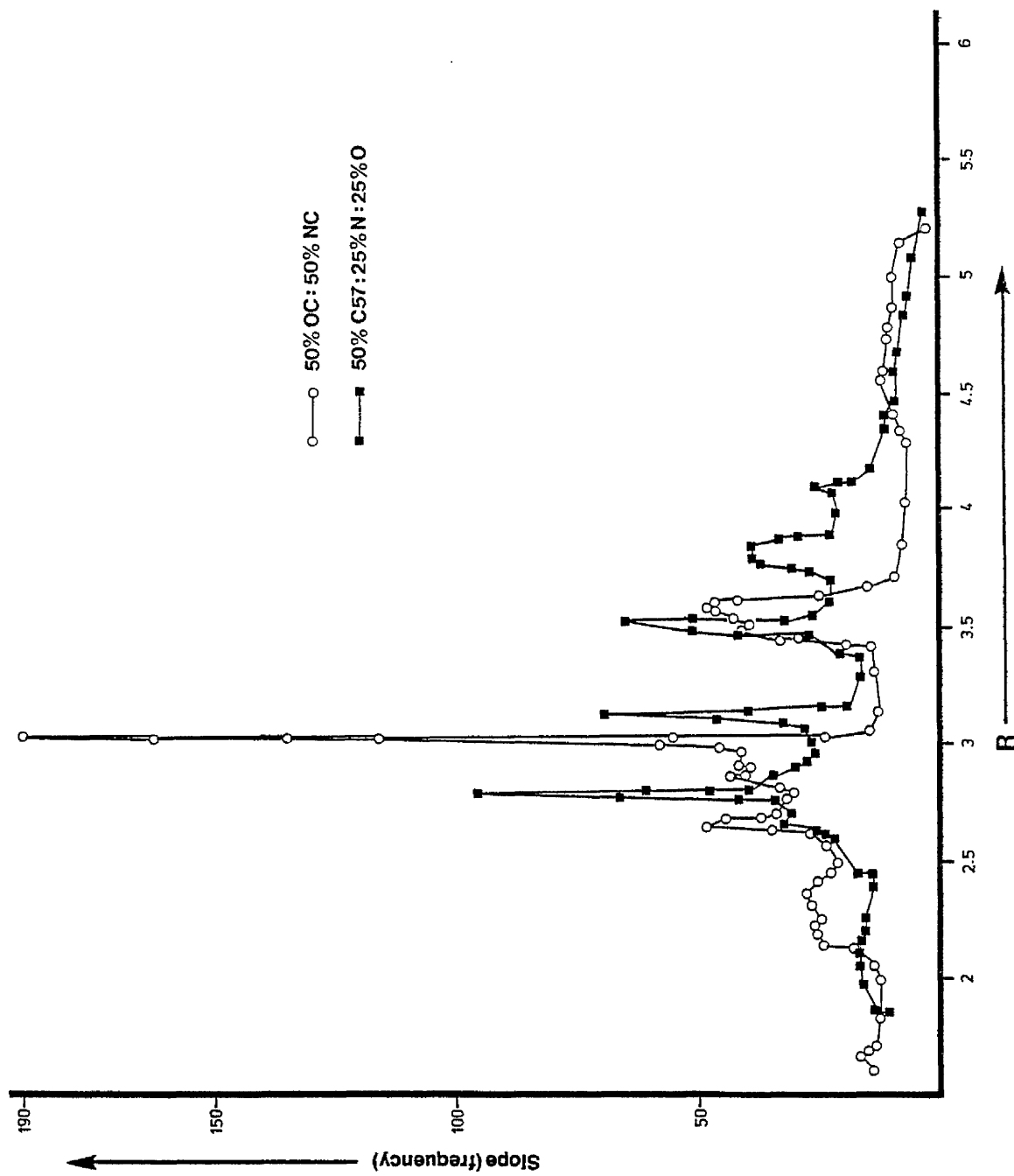


Figure 5.11 50% OC : 50% NC and the one-gene composite distribution; 50% C57 : 25% N : 25% O, both corrected for maternal influence.

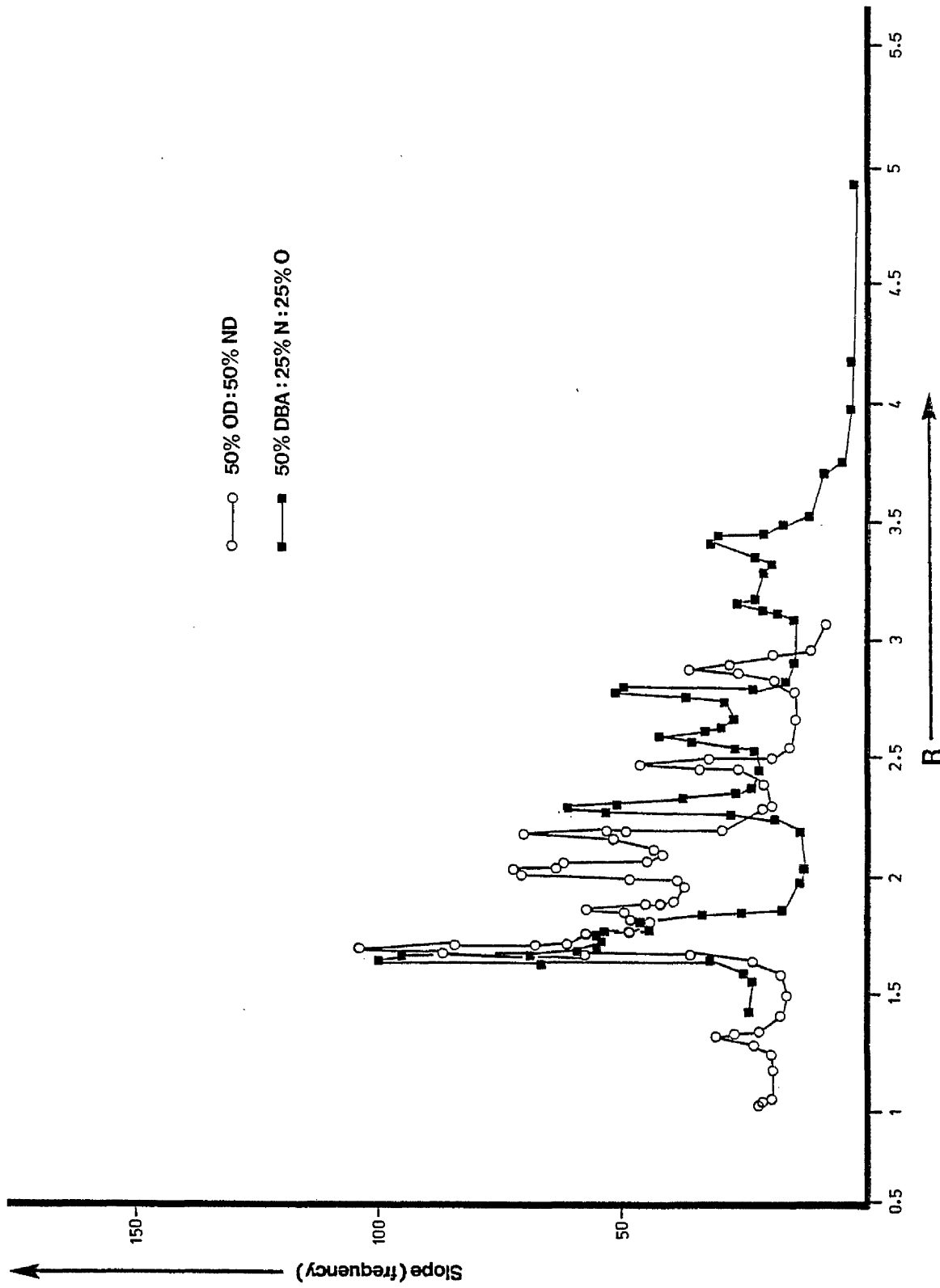


Figure 5.12 50% OD : 50% ND and the one-gene composite distribution; 50% DBA : 25% N : 25% O, both corrected for maternal influence.

Figure 5.11 indicates that, although statistically similar to it, the pooled backcross, 50% OC : 50% NC, possessed the rudiments of additional phenotypic classes on either side of the model distribution.

The significant difference between the DBA pooled backcross and its single locus model appears (Figure 5.12) to consist of extra classes found in the intervals $R = (0-1.5)$ and $R = (1.8-2.2)$. The total number of individuals occupying these classes was 35 out of a total of 68 (51.5%), suggesting a further gene difference differentiated the observed from the single locus distribution.

As the marker gene evidence has shown that there was considerable overlap between genetic classes (probably due to the large functional component of the phenotype) it was not possible, without larger sample sizes, to correlate them assuredly with phenotypic classes observed in the frequency distributions. If the four classes observed in OD (two are marked by smaller peaks) and ND (four distinct peaks) distributions, (Figure 5.10), are considered as approximate indicators of genetic segregant classes, the combined frequency distributions of 21 : 24 : 24 : 24 may suggest the presence of the 1 : 1 : 1 : 1 distribution expected for a two-locus difference.

5.9 CONCLUSIONS

In order to consider genetic and developmental aspects of the specific phenotype, R (the one hour maximal response to ACTH of one viable cell), it is appropriate, first, to discuss the importance of differences between its expressions in individual and pooled adrenal

preparations, as well as any inconsistencies between normal and corrected values.

In nearly every case, corrected inhibited R values appeared to be statistically compatible with normal values; some variance distortion, only, being evident among corrected NC values, while the statistical difference between both sets of CBA data has been suggested to be misleading on account of low sample size and the bimodal nature of the phenotype. Conclusions presented here, therefore, have been based on the analysis of all data except where a conflict with normal data was not readily explicable on the grounds of sample size, alone.

The mean corticosterone production values of single adrenal pairs were reduced for all strains compared with the rates observed using pooled organs. The causative agent(s) of this effect appeared to interact to a greater extent with cells from relatively lipid replete adrenals. The slightly increased vigour of the dissociation technique for individual adrenal pairs (section 3.2.3) may have had a more traumatic effect on the large lipid-filled cells more prevalent in replete adrenals. Equally, if the steroidogenic rate was determined, to some extent, by cells present in the highest functional state, perhaps by cell cooperation, the mean outputs of pooled organ preparations might tend to be polarised to the upper extreme of the functional spectrum revealed by individual non-segregant data.

Despite these factors, the differences between the parental and F_1 phenotypes, observed using pooled adrenals, were conserved for individual adrenal pairs; their means being identical in terms of rank and statistical significance except that no significant

difference was found between DBA and Q (F_1 CBA dam x DBA), undoubtedly due to the small sample size of Q.

Evidence of genetic differences, accounting for the phenotypic differences between DBA and C57 corticosterone production rates, was revealed in backcrosses in two ways.

Using F ratios as approximate measures of significant difference in variance, most backcrosses were more varied than parental lines in their distributions of individual R values. The significantly greater variance of QD (backcross of F_1 CBA x DBA to DBA), compared with that of DBA, suggested gene difference(s) also existed between CBA and DBA, although the diminished variance of QB (backcross to CBA) individuals (probably as a result of all the values being corrected) and the reduced sample size of F_1 Q individuals probably accounted for the lack of any F test significance involving other DBA/CBA hybrids. Such evidence could only be regarded as suggestive as the variances were not obtained from normally distributed values.

Definitive evidence of genetic differences, between DBA and C57, was obtained from the observed co-segregation of the genetic markers, Idh-1 (chromosome 1) and b (chromosome 4), with R value.

The half reciprocal backcrosses NC and CN possessed very similar distributions of individual R values which indicated the unlikelihood of X- or Y- linked effects. No information was obtained in this respect concerning differences between DBA and CBA.

The means of the reciprocal F_1 's, O (DBA dam x C57) and N (C57 dam x DBA), were different by 32% of their joint mean, implying, in the absence of sex chromosome-linkage, the operation of some maternal effect. The persistence of a 14.2% difference between the

means of half reciprocals, in backcross to DBA as well as to C57, indicated that the maternal effect was not derived from differences in maternal genotype (F_1N females being genetically identical to F_1O females) but rather from differences in functional states of dams. Some aspects of the maternal physiology or behaviour appeared to imprint itself negatively on offspring such that males (and presumably females) exhibited alternating deviations from rates hypothetically determined by their genotypes, alone.

A genealogical study of inbred parental strain (normal and corrected) values provided more support for the reciprocating nature of this effect. In Table 5.21, R values from two DBA and one C57 family, obtained from siblings, cousins and uncles of mice used for breeding, are given in a series of generations that were subject to the maternal effect indicated.

When all the repressed generation values were tested against stimulated values, the M-W 1-tail probabilities listed in Table 5.22 were obtained.

When families were considered separately, C57 values showed a significant difference between pooled values of alternate generations.

When the values of DBA families, A and B, were pooled according to their repressed or stimulated maternal effects, an overall comparison indicated that this strain also alternated, functionally, between consecutive generations.

Finally, a test between all C57 and DBA values of one generation type, and all those of the other, showed an increased significance.

Table 5.21. R values of individual male DBA and C57 decedents.

Maternal effect on generation	DBA (family A)	DBA (family B)	C57
Suppressive		<u>1.43</u> , <u>1.67</u> , <u>1.65</u> , 1.66, 1.65, 1.58, 1.64	
Stimulatory	2.57, 2.74, 1.43	No data	2.77, <u>4.08</u> , <u>3.86</u> , <u>3.00</u> , 2.44
Suppressive	2.25, 1.56, 1.40	1.64, 1.84, 1.72, 2.59, <u>3.44</u>	3.61, 2.75, <u>3.44</u> , <u>2.55</u> , <u>1.85</u> , <u>2.09</u> , 2.75, 3.48
Stimulatory	2.53, 2.02	2.29, 1.75, <u>1.73</u> , 2.34, 2.66	3.52, 3.55, 4.45, 5.08, 4.40, 3.85, <u>3.79</u>
Suppressive	1.91	2.37, 2.79, 2.77, 2.29	<u>2.53</u> , <u>3.95</u> , <u>3.73</u> , <u>2.93</u> , <u>4.09</u> , <u>4.35</u> , <u>1.55</u> , <u>2.10</u> , <u>2.58</u> , <u>4.59</u>
Stimulatory		<u>2.52</u> , <u>2.63</u> , <u>2.33</u>	<u>4.84</u> , <u>2.87</u> , <u>2.06</u>
Suppressive		1.73	

(Normal values are underlined)

Table 5.22. Mann-Whitney single-tailed probabilities for tests between all stimulated and all repressed data.

Family	M-W 1-tail probability (%)
DBA (A)	9.5 (NS)
DBA (B)	5.2 (just NS)
DBA (A + B)	2.4
C57	3.5
DBA + C57	1.0

Lack of data prevented seasonal effects from being eliminated as causes of such oscillations between generations, although, as will be discussed in Chapter 6, maternal effects could, potentially, assist in the timing of circannual rhythms.

Alterations in maternal endocrine function, whether drug-induced, or of genetic or environmental origin, have been known, for many years, to affect, permanently, the homeostatic mechanisms of offspring. In man, hyperthyroidism in mothers has been shown to result in hypothyroid children (Man et al., 1958). In experimental animals, parathyroidectomy has been shown to exert a maternal influence lasting at least four generations (Fujii, 1978). The development of adult sexual characteristics has been shown to be modifiable by perinatal influence. (Harris, 1964; Levine and Mullins, 1966). Anxiety induced in pregnant mice by overcrowding (Keeley, 1962), as well as stress hormone injection (Lieberman, 1963), was shown to result in reduced anxiety responses in offspring.

Although post-natal events of newly born rats were found to alter the subsequent development of the adrenal axis (Levine, 1962, Levine and Mullins, 1966), cross-fostering, although having some effect, indicated that pre-natal influences were stronger with respect to the development of anxiety responses in albino mice (Keeley, 1962). This implied the trans-placental passage of hormones, particularly corticosteroids; an assumption that was later vindicated by studies in rats (Zarrow et al., 1970) and mice (Nguyen-Trong-Tuan et al., 1971).

Suppression of foetal adrenal axis development was demonstrated in the rat, by injection of corticosterone into the pregnant mother

(Paul and D'Angelo, 1972; D'Angelo et al., 1973). Maternal adrenal hyperfunction was found to have a similar effect and adrenalectomy resulted in enhanced foetal adrenal activity (Milkovic et al., 1973a; Dupouy et al., 1975; Klepac et al., 1977; Klepac and Milkovic, 1979). Such alterations in the adrenal axis of the dam were observed to result in significant changes in adrenal weight at birth and, in the case of maternal hyperfunction, significantly reduced stress responses in adult 70-day old offspring (Milkovic et al., 1976), although no such permanency of effect was observed when foetal and neonatal rats had their adrenal function suppressed using dexamethasone (Grota, 1970).

Genetically determined characters such as the corticosteroid metabolism rates, both in mother and offspring, and foetal sensitivity determine the extent of expression of a maternal effect; susceptibility to cleft palate appearing to be largely dependant on foetal genotype (Marask et al., 1971). With respect to mouse open-eye mutants, only some of these are correctable by corticosteroid injection of pregnant mothers (Ricardo and Miller, 1967) and this has been shown to accompany high foetal corticosterone uptake from the maternal circulation in one case, but not in another (Nguyen-Trong-Tuan et al., 1971).

As the present parental strain evidence of maternal effect indicated that suppressed and stimulated values differed by 13.8%, in DBA's, and 19.8%, in C57's, of their common means, the penetrance of the effect appeared to be similar in both genotypes.

In the absence of cross-fostering, it was impossible to separate post-natal from intra-uterine influences. No observations were

made with regard to rearing behaviour beyond the fact that DBA's tended to be anxious mothers that resorted to infanticide and cannibalism more than other strains. It was thought, however, that the level of maternal plasma corticosterone was likely, in view of the evidence described, to be a major component of the maternal influence.

Only a small amount of information was obtained concerning female adrenal corticosterone production. The animals concerned were not pregnant and were of unknown oestrus state. The following table shows a comparison between four CBA individuals of each sex from one experiment, and also values obtained for pools (two adrenal pairs each) of DBA and C57 female adrenal cells:

Table 5.23. R values of individual virgin CBA females and of pooled DBA and C57 female adrenals.

Individual CBA Adrenal Pairs		Pooled Adrenal Pairs (2)	
Females	Males	Females	
		DBA	C57
4.41	2.68	2.52	3.37
4.21	2.50	(both corrected values)	
4.51	1.84		
5.60	3.87		
Means:	4.68	2.72	Female/Male = 1.72

The CBA female values, which were higher than any observed for all males (13), appeared to be similar to C57 females, which have previously been recorded as being about 1.5 times the male values

(Nandi et al., 1967). This result contrasts with that observed using radio-labelled progesterone precursor (Badr and Spickett, 1965) which was found to be converted to 11-deoxycortisol, cortisol and corticosterone at similar rates in CBA males and females.

The evidence suggesting higher corticosterone synthesis in C57 than in DBA females was in keeping with indirect evidence concerning their adrenal lipid contents. As it has been shown that the adrenal cholesterol ester sexual dimorphism is largely androgen-controlled in DBA's and oestrogen-controlled in C57's (Stylianopolou and Clayton, 1976a, 1976b), it was thought that lipid-replete female DBA's would be likely to have adrenals that produced corticosterone at similar rates to those of C57 males; lacking the oestrogen induced increase present in C57 females. If other genetic difference(s) besides that at the ald-2 locus are present, the phenotypic difference between females could be even greater.

The simplest hypothesis using the evidence contained in this chapter, concerning the possible mechanism of the maternal influence, is one that relates differences in maternal plasma levels of corticosterone during, and perhaps after, pregnancy with varying degrees of imprinting of the foetal adrenal axis. Many hormones, especially peptides, have been shown to exert a negative effect on target cell manufacture of their own receptors (Catt and Dufau, 1977). It is possible that foetal differences in plasma corticosterone levels, derived in the main from maternal differences, could result in altered corticosterone receptor numbers in the hypothalamus and pituitary. A suggested negative feedback effect of corticosterone on the adrenal

cortex, itself, (Mulrow, 1972) could be altered in a similar way. The positive maternal effect manifest in a study of adrenal weight (Meckler and Collins, 1965) may reflect a dichotomy between trophic and steroidogenic pituitary functions, or a distinct adrenal weight maintaining mechanism (Chester Jones, 1957).

Although direct effects were probably the major components of the maternal effect, it was likely that similar maternal/foetal interactions, in the development of other endocrine and nervous tissue, could contribute, indirectly.

Turning, now, to genetic effects, it will be shown that these were modulated in their penetrance by the non-genetic influence just described.

Considering the number and type of gene differences, first; it seemed probable that two major genes differentiated DBA and C57 corticosterone productive capacities. The apparent co-segregation of two markers, b and Idh-1, and also the significant departure of the combined 50% OD : 50% ND distribution from a one-gene model expectation, after reduction of maternal effects, formed strong evidence of this.

The approximate unimodality of overall shape of backcross distributions suggested genes were transmitted in coupling phase, and the similarity of the average F_1 mean, $\frac{\text{mean (O)} + \text{mean (N)}}{2} = 3.259$ R units), to the mean of C57, (3.456 R units), suggested that both loci were recessive in DBA.

The location of b on chromosome 4 (Green, 1966) indicates that a gene influencing corticosterone production may lie near other loci controlling endocrine (db - diabetes; Hummel et al., 1966; and

Bfo-bell-flash ovulation; Eleftheriou and Kristal, 1974), behavioural (Exa-exploratory activity; Oliverio et al., 1973) and neurological (asp-audiogenic seizure prone; Collins and Fuller, 1968) functions. It is also possible that adrenal control could be a pleiotropic effect of one of these established loci or of the b locus, itself. Some evidence exists (Shire, personal communication) that hepatic glucose-6-phosphate dehydrogenase activity, linked to corticosterone catabolism, appears to be associated with a coat colour gene.

The nature of the other gene, identified by Idh-1 as being on chromosome 1 (Henderson, 1965), was more certain on account of some observations, concerning adrenal colour, made on experimental mice. The subjective assignment of adrenals to three colour classes, reflecting total lipid content, enabled rough correlations between stored cholesterol ester and Idh-1 type, as well as corticosterone production (R), to be made, as shown in Table 5.24.

Adrenal colour scoring was less efficient among more lipid-depleted organs; DBA backcrosses colour classes being more equivocal than those of backcrosses to C57. This probably accounted for the greater significance of correlation of OC Idh-1 type with adrenal colour. Similarly, the general observation that OD and OC adrenals tended to be redder (more lipid-depleted) than ND and NC, was reflected in OC, but not OD, colour scores.

OC mice with intermediate lipid stores consisted of a significantly greater number of Idh-1 heterozygotes than homozygotes, also individuals with replete lipid stores contained a significantly larger frequency of Idh-1 homozygotes.

Table 5.24. Adrenal colour, Idh-1 type and corticosterone production (R).

All probabilities expressed as %'s									
Adrenal colour:		Red	Pale Red/Orange		Pale Orange/Yellow				
Adrenal lipid:		Depleted	Intermediate	fast/slow	het.	chi-square	slow	het.	chi-square
		fast	het.	chi-square	fast	het.	slow	het.	chi-square
OD	9	5	1.14	6	16	4.54*	-	-	5.0*
ND	12	9	1.00	11	11	0.00	-	-	NS
OC	-	-	-	5	19	8.16	16	5	1.0
NC	-	-	-	7	6	0.08	9	9	NS

* probability of chi-square value indicated

Adrenal colour versus R value			
Common Group	Group	greater than	Group
			M-W 1-tail p
OD	intermediate (28)	depleted (23)	NS (8.38)
ND	intermediate (26)	depleted (23)	NS
OC	replete (21)	intermediate (23)	0.50
NC	replete (21)	intermediate (23)	NS
depleted	ND (23)	OD (23)	4.36
intermediate	ND (26)	OD (28)	NS
replete	NC (21)	OC (21)	NS
intermediate	NC (13)	OC (23)	NS (11.12)

OD mice also demonstrated a significant predominance of Idh-1 heterozygotes among individuals of intermediate lipid type.

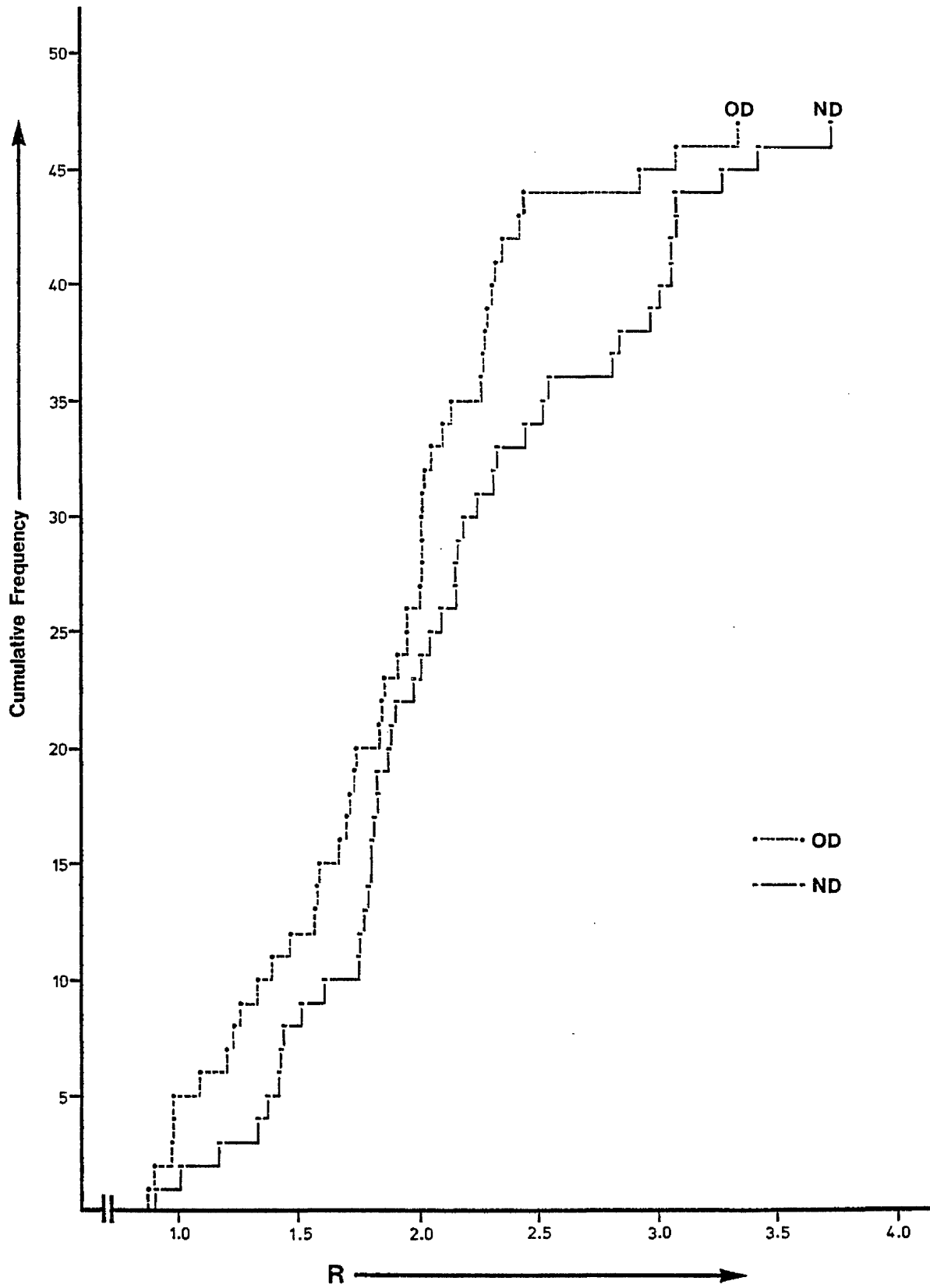
Likewise, corticosterone production in OC mice was significantly correlated with extent of lipid stores and a similar relationship was observed in OD individuals, although it was just not significant.

Further analysis of differences in corticosterone production in relation to lipid content, between pairs of reciprocal backcrosses, gave a similar result to that observed for Idh-1 (section 5.8.1.2.1); depleted (or Idh-1 fast) rather than intermediate (or Idh-1 het.) lipid content being the subclasses significantly responsible for differences between ND and OD values.

The fact that adrenal colour v. Idh-1 type, R v. Idh-1 type, and R v. adrenal colour all appeared to show significant effects only in backcrosses with O dams suggested that the Idh-1 locus, or one linked to it, exerted its effect on corticosterone production by alteration of adrenal lipid content.

A potentially rate-limiting character such as extent of cholesterol ester precursor stores is also implied by consideration of interactions between maternal influences and genetic segregant classes. In Figure 5.8 (p.172) it can be seen that the difference between OC and NC cumulative frequency distributions is moderately constant; the two graphs being roughly parallel and differing only in location. Similar comparison of OD and ND distributions (Figure 5.13), however, indicates a difference in shape as well as position of the two graphs; individuals at the high end of the OD distribution being markedly different from their ND analogues. An interpre-

Figure 5.13 Cumulative frequency distributions of ND and OD (all values).



tation of this could be that maternal influences appear to affect all segregant classes in a similar manner in backcrosses to C57, whereas, in those to DBA, certain genotypes are more affected than others. Some evidence has been presented already that maternal effects appear to operate to similar extents in C57 and DBA parental lines, making it unlikely that such differences between OD and ND were due, primarily, to segregation of genes controlling the maternal influence, itself. It was more likely that the pronounced interaction of genotype with maternal effect in DBA backcross involved some gene that became rate-limiting when homozygous for the DBA allele. This may have explained the fact that individuals that were homozygous for Idh-1 were significantly different corticosterone producers between OD and ND, whereas heterozygotes were not.

The DBA locus ald-2 has not been shown, irrefutably, to be unlinked or non-allelic to the AKR locus, ald-1. Complementation studies (Doering et al., 1973), did not include progeny tests that would have eradicated the possibility of different phenotypic classes being purely functional and similar to bimodalities found with R value in the present work, and with lipid content in another study of C57 and DBA adrenals (Wahlstein and Anisman, 1976). In the present work it was possible, therefore, that the Idh-1-linked gene was, in fact, the ald-2 locus which would then be linked or allelic to the ald-1 gene already mapped to the same chromosome (Taylor and Meier, 1976). It is also possible the Idh-1-linked locus may have a modifying function, on the expression of ald-2, only revealed by different maternal environments suggesting that adrenal lipid depletion may be directed by at least two genes, in both AKR's

and DBA's, when all components of inheritance are considered. It is possible that the previous assertion that the expression of ald-2 in DBA's was not rate-limiting with respect to steroidogenesis (Doering et al., 1973) was only partially true as examination of reciprocal backcrosses might have shown.

Maternal influences appeared to operate in opposite directions with respect to expression of the Idh-1- and b-linked genes; the former being significantly correlated with R in offspring of O dams while the latter appeared to influence steroidogenesis only in ND adrenals. Although the b-linked locus may have interacted separately with the maternal influence it seemed probable that this difference was more likely to have resulted from an interaction between the two genes. The Idh-1-linked gene probably exerted a rate-limiting effect in OD's which was epistatic to the expression of the b-linked gene. Such an interaction may have been indicated by the fact that, of the four combinations of Idh-1 and b types, only individuals homozygous for Idh-1 and heterozygous for b had significantly different R values between OD and ND.

To clarify; a major intra-adrenal target of maternal influences appears to be the expression of a locus on chromosome 1 which either controls or modifies adrenal lipid content. The resultant of the interaction between a negative maternal influence (suppression of adrenal activity) and this gene, when present in DBA homozygous form, appeared to be rate-limiting with respect to R value. When such an effect was marked, as in OD mice, it may have resulted in the reduced penetrance of a gene on chromosome 4 whose C57-derived allele appeared to be responsible for high corticosterone production

in ND mice; a maternal effect having ameliorated the expression of the gene(s) causing lipid depletion.

The non-genetic component of inheritance has appeared to be as important as the genetic with respect to corticosterone production. It is probable that, in the absence of reciprocal cross data, any genetical study of endocrine processes could be misleading.

CHAPTER 6

STRAIN DIFFERENCES IN THYMUS, SPLEEN AND TESTIS
WEIGHTS AND THEIR RELEVANCE TO ADRENAL FUNCTION6.1 INTRODUCTION

Having considered differences in adrenal corticosterone production, in vitro, in previous chapters, their relevance will now be considered in the wider context of their relationships with the weights of lymphoreticular target tissue and also testis size. This should enable the probable contributions of adrenal cortex function to the overall biology and disease susceptibility of each strain to be considered.

Although the lymphocytolytic action of adrenal corticoids has been established for some years (Dougherty and White, 1945; Baker et al., 1951) it is only relatively recently that the more subtle implications of such interaction have been explored; both in terms of which limbs of the immune response are most affected and in what ways. Although the thymus is now recognised to be highly sensitive to both glucocorticoids and sex steroids (Ahlqvist, 1976) the response of the spleen is less marked and, therefore, open to debate.

Splenic atrophy has been induced in mice, rabbits (Dougherty and White, 1945) and rats (Baker, 1951) by ACTH or steroid injection. A comparison of Peru (thought, for many reasons, to have highly active adrenal glands) with CBA mice indicated that the spleens of the former strain were not only smaller but were possibly less sensitive to ACTH-induced weight reduction, than the latter (Shire and Stewart, 1972). On the other hand, adrenal hypertrophy in response to long-term environmental differences has been observed to be accompanied by splenic growth

irrespective of mouse strain (Mos, 1976). The implication of growth hormone as probable cause, in that particular case, serves to emphasise the complexity of endocrine functions which are seldom completely separable. Similarly, the thymus has been thought to exert a hormonal effect on adrenal maturation (Pierpaoli and Sorkin, 1972), a hypothesis refuted in favour of an indirect effect, mediated by the pituitary (Shire and Pantelouris, 1973). More recently, however, a direct influence of two different thymic extracts on in vitro rat adrenal corticosterone production has been observed (Deschaux et al., 1977).

Some of the original evidence for strain differences in lymphoreticular cell sensitivity to corticosteroids was revealed in the search for reliable bioassay techniques. Some strains, such as C57BL/6 were found to develop eosinopenia rapidly in response to steroid injections whereas others, such as DBA/1, were found to be relatively insensitive; eosinopenia developing more slowly (Wragg and Speirs, 1952; Thiessen and Nealey, 1962). Differences in thymus size have been observed between different strains of rat (Bellamy et al., 1976) and mouse (Metcalf, 1960; Albert et al., 1965; Simpson, 1973). A gene, Hom-1, linked to the H-2 locus in mice, was thought to be responsible for a broad array of target organ sensitivities to androgen (Ivanyi et al., 1969; Ivanyi et al., 1972a). The H-2a haplotype was found to be associated with heavier testes, thymuses and lymph nodes, but lighter vesicular glands, compared with organs from the congenic H-2b strain of C57BL/10. A subsequent study, (Pla, 1976) revealed that the H-2a haplotype was also associated with greater sensitivity to corticosterone-induced thymocytolysis, in vivo and in vitro.

The importance of the thymus as an immune censor preventing the formation of 'forbidden clones' of immunocytes (Burnet, 1959) has been reinforced by more recent evidence that a corticosterone-sensitive sub-population of mouse thymic lymphocytes (positive for TL antigen, high theta antigen) may be the products of a sterile differentiation pathway designed to ensure the death of potentially self-reactive clones within the thymus (Shortman and Jackson, 1974). This role of the thymus has also been revealed by an examination of the immunology of the thymusless nude mouse, in which the wasting disease has been thought to consist of cross-reactive immunity stimulated by environmental pathogens, and which is characterised by high ratios of immunoglobulin M:immunoglobulin G (Pantelouris, 1973b). Similar high IgM:IgG ratios were found in C57BL mice, which were suggested as being symptomatic of the premature immunological senescence and auto-immune diseases found in this strain (Pantelouris, 1974). The similarity in thymic lymphocyte composition between young cortisol-treated, and untreated, old C57BL mice (Bellamy and Alkufaishi, 1972), may indicate a causal relationship between the high adrenal corticosterone productive capacity and the development of immune defects found in this strain. A recognised harbinger of anti-immune disease, anti-nuclear factor, was found to be present in sera of C57BR(6/13) and C57BL(1/16) but not in those from DBA/1 (0/8), DBA/2 (0/10) or CBA(0/9) mice raised under conventional conditions (Barnes and Tuffrey, 1967). The high incidence of leukaemia in AKR, rather than C3H, mice was thought to be due, in part, to reduced adrenal function as the thymuses of this strain appeared to be highly sensitive to the action of glucocorticoids (Metcalf, 1960). Large thymus has also been related to low mammary carcinoma incidence (Belyaev and Gruntenko, 1972) although in this case an adrenal, rather than a directly immunological explanation,

seems likely in view of the stimulatory effects of adrenocorticoids on mammary tumour virus function (Lee et al., 1981).

It was originally demonstrated that cortical lymphocytic migration into the thymus medulla was accompanied by differentiation to immunocompetence and also by loss of sensitivity to corticoid treatment (Weissman, 1973). Although rat thymus cells in suspension appeared to lose dexamethasone-binding capacity upon immunological transformation (Cidlowski and Munck, 1976), another study transforming cultured human lymphocytes with the same mitogen resulted in dexamethasone binding that was related to mitotic rate (Smith et al., 1977). Genetic variants for dexamethasone binding by lymphoma cells indicated the possibility of lesions at many sites being able to result in steroid insensitivity (Sibley and Tomkins, 1974).

Apart from the dramatic influence of androgens on the expression of ald genes (Molne, 1968; Doering et al., 1972), differences in testis and target organs have been observed between DBA/2J and C57BL/10J strains (Shire and Bartke, 1972; Bartke and Shire, 1972) and between many other strains (West et al., 1980), although the small testes produced by a Y-linked factor in CBA mice (Hayward and Shire, 1974) were not related genetically to the testicular hypoplasia of C57BL's. Pathological mutants located on the X chromosome have been observed in mice (Lyon and Hawkes, 1970) and in humans (Meyer et al., 1975) that result in androgen insensitivity.

The antagonism between adrenal and testis appears to be mutual as cortisol depresses testosterone production in humans (Schaison et al., 1978), although ACTH has been shown to increase plasma testosterone levels, in rabbits, probably by increasing testicular blood flow (Fenske, 1980), and also to stimulate 11-beta-hydroxylation in human

testicular tumours, and in normal rat testes (Villemee and Fisher, 1975).

Androgens, like corticosteroids, also appear to be important in controlling thymus function, a role that is highlighted by its apparent deficiency in NZB and NZB hybrid mice. Although the autoimmune disease in these mice is complex; being characterised by antibodies to erythrocytes as well as thymocytes (Raveche et al., 1978; Steinberg et al., 1980) the main underlying defect appears to consist of the loss of an androgen-sensitive thymus suppressor function that allows the production of anti-T cell antibodies (IgM immunoglobulins) (Steinberg et al., 1980). The male F₁ hybrids, produced by crossing NZB with DBA/2J mice, appear to be markedly less affected by the autoimmune syndrome indicating the possibility of DBA's possessing lymphoreticular systems that are highly sensitive to androgen (Raveche et al., 1980). Genetic analysis, using recombinant inbred lines derived from crosses between NZB and DBA/2J, CBA and ALN, have discovered many recombinant classes in which single features of the disease segregate separately, including androgen suppressibility (Raveche et al., 1980a).

Thymic hormone extracts (Goldstein et al., 1970) have been shown to inhibit autosensitisation in vitro and in vivo (Small and Trainin, 1975; Carnaud et al., 1975) and also to prolong the effectiveness of suppressor T cells in NZB thymuses in their inhibition of antibody formation to a thymus independent antigen (Bach and Niaudet, 1976), although their administration to NZB mice did not appear to significantly alter the course of the disease (Gershwin et al., 1976).

Defects in responsiveness are not solely confined to the thymus; an inability to respond to type 3 pneumococcal polysaccharides has been located to an X-linked gene in CBA/N mice (Scher et al., 1976; Cohen et al., 1976). An autosomal dominant gene, Dh, that causes total lack of spleen development, has been shown, when placed in NZB/W F₁ hybrids, to prolong male, but reduce female survival (Gershwin et al., 1980), an effect which suggests the syndrome could develop through qualitatively different immune mechanisms in high and low androgen environments.

From these, and complexities of interaction outlined earlier, it is inconceivable that either natural selection in wild mice, or artificial selection of inbred lines, should operate upon endocrine characters, in isolation. Unless an animal is distinctly pathological and would not survive or reproduce except by artificial means (e.g. maintenance in specific pathogen free conditions improves the life-span and fertility of nude mice, Pantelouris, 1973b) it must possess a balanced physiology conferring reasonable chance of reproductive success even if it possesses genes that give rise to 'abnormal' function, in one of the major endocrine glands. Such success has been vouchsafed in C57BL's by their co-adaptation of seminal vesicles of heightened sensitivity to androgen in the face of low plasma levels (Bartke, 1974). In the case of AKR mice, although bordering on the pathological, the thymus has evolved extra sensitivity to corticosterone as a means of offsetting the effects of reduced adrenal function (Metcalf, 1960). In the course of this chapter an examination of differences in lymphoreticular target organ weights in relation to the in vitro measure of adrenal corticosterone productive capacity, R, and testicular weight, in crosses between DBA, CBA and C57 mice, could be expected to reveal evidence of such co-adaptive evolution.

Genetic factors, linked to the DBA-derived alleles of either the b or Idh-1 loci, might be expected to be found to increase sensitivity in lymphoreticular tissue to corticosterone, as compensation for the low adrenal steroid production associated with these alleles, observed in the previous chapter. Not necessarily the same backcrosses might, by the same logic, betray segregation of factors governing the androgen-responsiveness of thymus and spleen.

If the maternal effect, noted already in relation to adrenal corticosterone production, is found to alter the expression of genes controlling target organ sensitivity to a particular steroid, some indication of the contribution of such an effect to the overall fitness of the animal should become evident. A marked difference between the reciprocal F_1 hybrids of NZB x NZW and NZB x DBA/2 mice has already been observed both with respect to thymus development (Simpson, 1973) and antibody production (Raveche et al., 1978).

Following brief sections describing statistical methods and correlations of organ weights and corticosterone productive capacity (R) with body weight, three sections will be devoted to strain comparisons for thymus, spleen and testis weights. Following this brief reference will be made to changes in the adrenal corticosterone production phenotype, R, with age. The correlations between organ weights and R will be considered in a penultimate section; the final section being devoted to a description of conclusions reached.

6.2 STATISTICAL METHODS

The following sub-sections include, first, some explanation of the general statistical strategy involved in the analysis of results presented in this chapter. Following this, two further sub-sections describe the non-parametric and parametric statistics used. Finally, a description is included of the correction of some spleen weight data known to have been obtained under different drying conditions.

6.2.1 General strategy

The distributions of many organ weights were found to be similar to those obtained for corticosterone production; tending to depart from normality through the presence of extra functional groupings. As some evidence was presented in the conclusion to Chapter 5 that functional dichotomies may exist in adrenal function expressed in alternating generations, it was felt that a prudent course, in analysing target organs, was to perform the back-bone of the analysis using ranking (non-parametric) techniques and overlaying this, where suitable non-parametric techniques were not available (e.g. partial correlation, for which the sampling distribution of the Kendall partial rank correlation coefficient is unknown), with the parametric equivalents. Under these circumstances, the additional information provided by the parametric techniques was regarded as descriptive rather than definitive.

The use of multi-factorial analysis of variance was impracticable due to imbalances in the numbers of representatives of strains in different age groups. The basic strategy thus consisted of the application of the Kruskal-Wallis one-way analysis of variance (K-W test),

firstly, within strains across age groups, then, secondly, within age groups across strains.

In Mann-Whitney tests, used to analyse within groups already tested by K-W test, significance levels were not divided by the number of tests carried out that were identical with respect to the laws of chance; this was felt to be unjustified as the comparisons differed so widely in sample size. This was regarded as a minor weakness but isolated probabilities near the 5% level were regarded with appropriate suspicion.

6.2.2 Non-parametric tests

Three tests, other than the Mann-Whitney test already described in Chapter 5, were used according to their description by Siegel, 1956; the Kruskal-Wallis one-way analysis of variance, the Friedman two-way analysis of variance and Spearman's rank correlation coefficient.

The various statistics were found in the following ways;

$$\text{Kruskal-Wallis statistic: } H = \frac{12 \times P}{N(N+1)} - 3(N+1)$$

Where: N = total no. of cases in pooled samples

P = sum of $\left(\frac{R^2}{n}\right)$ across samples where R = sum of ranks within a sample.

n = sample size.

R is computed, for each sample, by totalling the ranks of that sample from a gross ranking of all cases from all samples.

Correction for ties was performed by dividing H by:

$$1 - \frac{(\text{sum of } T\text{'s for all groups of ties})}{N^3 - N}$$

where $T = t^3 - t$, where t is the no. of tied observations within a tied group of values. H follows the chi-square distribution with

(the no. of samples - 1) being the degrees of freedom.

$$\text{Friedman statistic: } (\text{Chi-square})_r = \frac{12 \times Q}{N \times k(k+1)} - 3N(k+1)$$

Where: N = no. of rows, k = no. of columns

Q = sum of (R^2) across columns where R = sum of ranks within a column.

Samples, which have to be of equal size, are set out in a row by columns design (e.g. age group by strain) and rankings within rows are found, which are then totalled to give R for each column. The sampling distribution is that of chi-square, with $(k-1)$ degrees of freedom.

$$\text{Spearman rank correlation statistic: } r_s = 1 - \left(\frac{6M}{N^3 - N} \right)$$

Where: N = no. of pairs of observations

M = sum of d^2 where, having ranked each variable,

d is the difference in rank between variables in a pair.

Correction for ties was unnecessary as the distorting effect is only appreciable with many tied values.

Significance was determined either by referring to tables of critical r_s values (sample size less than 10) or by use of the t distribution (sample size greater than 10), with $N-2$ degrees of freedom;

$$t = r_s \times \left(\frac{N-2}{1-r_s^2} \right)^{0.5}$$

In future sections r_s will be referred to as $r(\text{NPAR})$.

6.2.3 Parametric statistics

Pearson's product-moment parametric correlation coefficient was calculated according to the formula:

$$r(\text{PAR}) = \frac{\text{covariance of } x \text{ and } y}{(\text{variance}(x) \times \text{variance}(y))^{0.5}}$$

The significance was obtained by the same method as that used for Spearman's correlation by ranks.

Regression equations for y on x were formed from the formula:

$$Y = \text{MEAN}_y + b(x - \text{MEAN}_x)$$

$$\text{where } b = \frac{\text{sum of } (d_x \times d_y)}{\text{sum of } (d_x)^2} \quad \text{in which } d_x \text{ and } d_y \text{ are the}$$

deviations of each value from the means of x and y.

Partial correlation coefficients, which measure the degree of correlation between two variables, having removed the effects of a third, were computed using the following formula:

$$r_{xy.z} = \frac{r_{xy} - (r_{xz})(r_{yz})}{(1 - r_{xz}^2)^{0.5} \times (1 - r_{yz}^2)^{0.5}}$$

Where r_{xy} , r_{xz} and r_{yz} are the zero order correlations.

When (n + 1) variables were controlled, the above formula was used except n order, instead of zero order, correlations were used.

6.2.4 Correction of spleen dry weights obtained under different drying conditions

A proportion of spleens were subjected, by accident, to a higher oven temperature of about 150°C, for a short time. Mann-Whitney comparisons between these and organs (derived from animals of comparable age) dried under normal conditions, indicated, in every case, a significant loss of weight.

Overheated values were corrected by regression analysis in the following way. Taken a strain at a time, ranked overheated spleens were compared with the ranked normal group. Corresponding mean rankings were used to form regression lines so that if, for example, 6 overheated were being compared with 12 normal values, the lowest ranked value of the former was paired with the mean of the lowest, and second lowest, ranked values of the latter, and so on for each remaining overheated value. Regression equations formed in this way were then tested for significant difference from one another by the analysis of variance method described by Draper and Smith (1966).

As might be expected with spleens that displayed strain differences in shape, DBA-like, F_1 -like and C57-like regressions were significantly different from each other, thus three regression equations were used to correct overheated values. Their slopes and intercepts are given in Table 6.1.

Table 6.1 Regression statistics used to correct overheated spleen weights

Pooled strains	n	slope	intercept
C57, OC	30	1.012	1.285
N, O and NC	22	1.622	-2.443
OD, ND and DBA	45	2.405	-6.493

In the section concerned with strain differences in spleen weight, a distinction will be maintained between all spleen data (including corrected values), and data obtained under normal drying conditions.

6.3 CORRELATIONS OF ORGAN WEIGHTS AND CORTICOSTERONE PRODUCTION (R) WITH BODY WEIGHTS

Tables 6.2 and 6.3 give the non-parametric and parametric correlation coefficients ($r(\text{NPAR})$ and $r(\text{PAR})$, respectively) for absolute organ weight versus body weight for males of each strain.

The difference between using non-parametric and using parametric correlation methods appears, from Tables 6.2 and 6.3, to be relatively trivial; there being only four dissimilar probabilities that mostly reflected the fact that the parametric test used more information and was, therefore, more powerful.

As organ weight and body weight both vary with age (thymus weight, for example, is known to sharply decrease with age) it was important to try and assess the true relationship between organ and body weight by eliminating age as a cause of spurious correlation between them. This was done by finding partial correlation coefficients for organ weight versus body weight, controlling for age. Table 6.4 gives these coefficients.

The partial correlations, shown in Table 6.4, appeared to vary both with organ and with strain. Thymus weight seemed to vary independently of body weight for all strains except one. This result confirmed an earlier observation that relative thymus weight might be of limited statistical value, for this reason (Simpson, 1973).

Testis weight, an androgen-dependent character, due to the stimulatory effect of testosterone on spermatogenesis (Shire and Bartke, 1972), appeared to be correlated to body weight to a greater degree than spleen weight. In CBA mice, and their crosses to DBA, the reduced correlations between spleen and body weight, as well as testis and body weight, may indicate a degree of androgen insensitivity.

Table 6.2 Non-parametric correlation coefficients for absolute organ weight versus body weight (BWT)

Strain	(All probabilities expressed as %'s)					
	THYMUSA versus BWT		SPLEENA versus BWT		TESTISA versus BWT	
	r(NPAR)	n	p	r(NPAR)	n	p
DBA	-0.3187	72	0.3	+0.1368	66	NS
CBA	-0.4152	35	0.7	+0.2990	35	4.0
C57	-0.3142	85	0.2	+0.4042	86	0.09
F ₁ (Q)	-0.0348	16	NS	+0.5586	12	3.0
F ₁ (O)	-0.3726	22	4.4	+0.0360	22	NS
F ₁ (N)	+0.3566	46	0.7	+0.2945	46	2.3
OD	-0.2230	47	NS	+0.3978	47	0.3
ND	-0.0786	38	NS	+0.6026	45	0.09
QD	-0.3129	23	NS	-0.1394	23	NS
OC	+0.1198	44	NS	+0.2246	45	NS
NC	+0.1059	38	NS	+0.5362	37	0.09
CN	+0.0157	22	NS	+0.2755	23	NS
QB	-0.0916	20	NS	+0.1424	20	NS

(Thymusa, spleena, testisa = absolute thymus, spleen, testis wts.)

Table 6.3 Parametric correlation coefficients for absolute organ weight versus body weight (BWT)

Strain	(All probabilities expressed as %'s)							
	THYMUSA versus BWT		SPLEENA versus BWT		TESTISA versus BWT		n	p
	r(PAR)	n	p	r(PAR)	n	p		
DBA	-0.3637	72	0.1	+0.1771	66	NS	67	0.1
CBA	-0.4308	35	0.5	+0.4150	35	0.7	35	NS
C57	-0.3418	85	0.1	+0.3690	86	0.1	83	0.1
F ₁ (Q)	+0.1471	16	NS	+0.3846	12	NS	13	NS
F ₁ (O)	-0.3712	22	4.4	-0.0695	22	NS	21	3.2
F ₁ (N)	+0.2991	46	2.2	+0.2081	46	NS	44	0.1
OD	-0.2528	47	4.3	+0.3371	47	1.0	47	NS
ND	-0.0680	38	NS	+0.6386	45	0.1	43	0.1
QD	-0.2789	23	NS	-0.0306	23	NS	22	3.8
OC	+0.1545	44	NS	+0.0951	45	NS	45	0.3
NC	+0.2921	38	3.8	+0.4898	37	0.1	38	NS
CN	-0.0808	22	NS	+0.3375	23	NS	23	0.1
QB	-0.1731	20	NS	+0.1263	20	NS	20	NS

(Thymusa, spleena, testisa = absolute thymus, spleen and testis wts.)

Table 6.4 Partial correlation coefficients (parametric) between absolute organ weight and body weight (BWT) controlling for age.

Strain	(All probabilities expressed as %'s) Controlling for Age											
	THYMUSA versus BWT		SPLEENA versus BWT		TESTISA versus BWT		THYMUSA versus BWT		SPLEENA versus BWT		TESTISA versus BWT	
	r(PAR)	n	p	r(PAR)	n	p	r(PAR)	n	p	r(PAR)	n	p
DBA	-0.1367	69	NS	+0.1623	63	NS	+0.6208	64	0.09			
CBA	-0.2258	32	NS	+0.1855	32	NS	+0.2941	32	4.6			
C57	+0.0133	82	NS	+0.2913	83	0.3	+0.4620	80	0.09			
F ₁ (Q)	+0.1720	13	NS	+0.4893	9	NS	+0.4083	10	NS			
F ₁ (O)	-0.1751	19	NS	-0.0258	19	NS	+0.7236	18	0.09			
F ₁ (N)	+0.4363	43	0.1	+0.4008	43	0.3	+0.5132	41	0.09			
OD	+0.1771	44	NS	+0.2471	44	4.9	+0.2130	44	NS			
ND	+0.1650	38	NS	+0.5964	42	0.09	+0.5352	40	0.09			
QD	+0.1098	20	NS	+0.2856	20	NS	+0.0219	19	NS			
OC	+0.1712	41	NS	+0.2264	42	NS	+0.3965	42	0.4			
NC	+0.1148	35	NS	+0.5849	34	0.09	-0.1709	35	NS			
CN	-0.0885	19	NS	+0.3294	20	NS	+0.6867	20	0.09			
QB	-0.1934	17	NS	+0.1405	17	NS	-0.1671	17	NS			

(Thymusa, spleena, testisa = absolute thymus, spleen and testis wts.)

Reciprocal crosses revealed differences in correlations with body weight, the most marked of which appeared to be that found between $F_1(N)$ and $F_1(O)$ with respect to thymus and spleen weights.

In order that some more light might be shed upon the influences of the adrenal and the testis on body weight, second order partial correlations were found between testis absolute weight and body weight, controlling for age and corticosterone productivity (R), and also between corticosterone productivity (R) and body weight, controlling for age and absolute testis weight, as shown in the following table:

Table 6.5 Partial correlation coefficients (parametric) between absolute testis weight (testisa) or corticosterone productivity (R) and body weight (BWT), controlling for age, and either R or testis, respectively. (All probabilities expressed as %s)

Controlled: Strain	Testisa v. BWT			R v. BWT		
	Age, R			Age, Testisa		
	r(PAR)	n	p	r(PAR)	n	p
DBA	+0.626	28	0.09	+0.105	28	NS
CBA	-0.031	9	NS	+0.552	9	3.9
C57	+0.494	28	0.3	-0.311	28	4.7
$F_1(O)$	+0.720	17	0.09	-0.329	17	8.4
$F_1(N)$	+0.473	29	0.4	-0.179	29	NS
CN	+0.685	19	0.09	-0.058	19	NS
OD	+0.208	43	8.5	-0.090	43	NS
ND	+0.536	39	0.09	+0.172	39	NS
OC	+0.314	41	2.0	+0.397	41	0.4
NC	-0.177	34	NS	+0.155	34	NS
QD	+0.017	16	NS	+0.109	16	NS
QB	-0.181	16	NS	-0.139	16	NS

(n = degrees of freedom)

The most significant correlations found in this way were those of the parental strains which all appeared to be qualitatively different with respect to adrenal or testis correlation with body weight. DBA appeared to have the strongest positive relationship between testis absolute weight and body weight, unlike CBA which lacked any such association. On the other hand, the latter strain possessed the strongest positive relationship between corticosterone productivity (R) and body weight, which, not surprisingly, in view of its low adrenal steroid output, was not emulated by DBA. C57 mice were apparently similar to DBA, with respect to testis correlation with body weight, but were the only strain of animals to demonstrate a significant negative correlation between adrenal corticosterone production, in vitro, and body weight. These facts, as well as the tendency for one reciprocal strain to betray stronger correlations for both testis weight and corticosterone productivity with body weight, compared with the other reciprocal strain, were thought to suggest possible pituitary (or brain) differences between strains.

The high positive correlation between testis absolute weight and body weight in CN mice, compared to OC and, particularly, NC, was thought to indicate the possibility that a C57-derived factor on the X-chromosome may be responsible for greater sensitivity to androgen, at least in respect of its control of body weight.

Unlike those observed in Chapter 5 with respect to corticosterone production, reciprocal differences in body weight correlations did not follow an identifiable pattern which was to be expected in view of the complex interactions between many endocrine tissues that govern its expression. This was displayed further when associations were sought between body weight and marker type.

The Idh-1 DBA-derived allele appeared to be linked to higher body weight in 11-13 week old ND's (M-W 2-tail $p = 0.0008$) but, in heterozygous form, to lower body weight in OC's in 15-17 week old animals (M-W 2-tail $p = 0.0042$) as well as in animals from all age groups (M-W 2-tail $p = 0.0258$).

This paradox was also reflected in comparisons between different Mod-1 and d alleles, which are on the same chromosome.

ND's possessing the Mod-1 or d DBA allele were lighter than heterozygotes for mice of all ages (M-W 2-tail p 's; 0.0308 and 0.045 respectively) whereas the Mod-1 DBA allele, in the heterozygous state, in OC's, was just not significantly linked with higher body weight, in animals of all ages (M-W 2-tail $p = 0.056$).

As Idh-1 has already been shown to be linked to factor(s) controlling lipid content and corticosterone productivity (R) in adrenals phenotypes which are known to be testosterone dependent, these paradoxical marker associations may be the result of the apparent difference in relationship between R and body weight noted in DBA's and C57's (Table 6.5).

Although absolute weights and relative weights (absolute weight/body weight $\times 10^2$) are analysed simultaneously in forthcoming sections dealing with strain comparisons, the information within the present section will assist in discriminating real organ-specific differences from those resultant from general body weight diversity.

6.4 STRAIN DIFFERENCES IN THYMUS WEIGHT

In this, and subsequent sections concerned with spleen (Section 6.5) and testis (Section 6.6) weight, the main lines of enquiry are contained in three main sub-sections concerned with: strain differences in weight (and change of weight) of organs, variance ratio evidence of genetic segregation, and, finally, the association of marker genes (Idh-1, Mod-1, d, b and a) with any organ weight differences.

The Kruskal-Wallis non-parametric one-way analysis of variance was used to detect differences between strains within 14-day age groups and also to indicate significant differences between ages within each strain. This information, as well as supportive evidence supplied by non-parametric, $r(\text{NPAR})$, and parametric, $r(\text{PAR})$, correlation statistics, as well as slope estimates for thymus weight v. age, are contained in the first Sub-section, 6.4.1. Comparison of variances of backcross and non-segregant thymuses will be described, in Sub-section 6.4.2, using ordinary variance estimates, and those formed from values centred to zero round each age group mean, in order to eliminate the component of variance caused, largely, by differences in thymic involution rate.

The final sub-section examines differences between groups selected for different marker types by consideration of their age-related changes, as well as their gross overall differences.

6.4.1 Strain differences in thymus dry weight within and across 14-day age groups

6.4.1.1 Differences between strains in thymic involution

The mean absolute and relative thymus weights are recorded for each strain, for each age group; 50-63, 64-77, 78-91, 92-105 and 106-119 days (abbreviated to their mean ages: 8, 10, 12, 14 and 16 weeks) in

Tables 6.6 and 6.7. Some older mice, indicated by 'a' and 'b', were used for CN and QD backcrosses. Thymus weight changes were assessed in three main ways (in their order in the tables); K-W tests were performed across age groups (p given as % in tables), non-parametric correlation coefficients, $r(\text{NPAR})$'s, were found for organ weights with age (to the nearest day) and, finally, parametric correlations, $r(\text{PAR})$'s, and slope estimates for the regression line were computed. Tables contain the value for $r(\text{NPAR})$ followed by the single-tail probability for this and its parametric counterpart, expressed as %'s multiplied by 100. The slope estimates are multiplied by a factor specified in each case. Mann-Whitney tests between adjacent age groups, within a strain were used to highlight the occurrence of dramatic weight changes, and the younger of two age groups, so tested and found to be significantly different, is indicated by asterisks denoting the degree of significance.

The general pattern of strain differences was similar for both absolute and relative dry weights although the negative correlations found in the former tended to be exaggerated in the latter, probably due to the artificial effect of the increasing body weight component, which has already been shown to vary independently of thymus weight, for these strains (Section 6.3). The similar negative correlations and slopes, of DBA and C57 thymus weights with age, belied differences in the timing of periods of maximum change. C57, on one hand, as well as possibly its backcrosses, OC, NC and CN (although early age period data is lacking, except in the case of CN) appeared to have stable thymus weights between 12 and 16 weeks; maximum change occurring earlier between 8 and 12 weeks. With DBA and its backcrosses, OD, ND and QD, the position was the exact converse; maximum weight loss occurring

Table 6.6 Mean absolute thymus dry weights for different strains and age groups

Age Weeks:	Absolute Weights (MGMS) $\times 10^2$					K-W Across Ages p (%)	r(NPAR)	p (% $\times 10^2$) NPAR, PAR	Slope ($\times 10^3$)
	8	10	12	14	16				
Strain									
DBA	871 (12)	909 (10)	893 (19)	719 (12)	619 (15)	0.2	-0.482	10, 4.0	-48
CBA	813 (15)	-	768 (5)	809 (8)	705 (4)	NS	-0.276	NS, NS	-12
C57	1204 (11)	1121 (20)	997 (14)	1005 (16)	912 (13)	0.5	-0.404	10, 0.8	-46
Q	798 (2)	962 (12)	1225 (2)	-	-	NS	+0.694	10, 144	+175
O	-	1148 (2)	1255 (5)	1011 (7)	945 (8)	NS	-0.498	90, 282	-105
N	-	1034 (4)	907 (17)	949 (19)**	664 (6)	NS	-0.231	NS, NS	-64
OD	1195 (.3)	1102 (6)	1234 (8)	1055 (10)**	805 (17)	0.6	-0.509	10, 5.8	-92
ND	-	1160 (5)	1144 (17)*	877 (13)	-	4.9	-0.454	20, 47	-141
OC	-	-	1264 (6)	1283 (19)*	1108 (19)	NS	-0.193	NS, 362	-84
NC	-	-	1013 (15)	1010 (13)	1006 (10)	NS	+0.054	NS, NS	-13
CN	-	1052 (12)	-	1131 (4)	1050 ^a (6)	NS	-0.028	NS, NS	+26
QD	-	-	1161 (11)*	816 (5)	821 ^b (7)	2.1	-0.524	50, 321	-48
QB	-	879 (10)	749 (6)	864 (4)	-	NS	-0.215	NS, 403	-28

* M-W 2-tail p less than 5% between this and adjacent older group.

** M-W 2-tail p less than 2.5% between this and adjacent older group.

^a These animals 19 weeks old.

^b These animals 24 weeks old.

Table 6.7 Mean relative thymus dry weights for different strains and age groups

Age Weeks:	Relative Weights $\times 10$ (mgms./100 gms. bwt $\times 10$)					K-W Across Ages p (%)	r(NPAR)	p (% $\times 10^2$) NPAR, PAR	Slope ₂ ($\times 10^2$)
	8	10	12	14	16				
Strain									
DBA	338 (12)	366 (10)	340 (19)	274 (12)*	218 (15)	0.09	-0.533	10, 0.6	-23
CBA	367 (15)	-	304 (5)	326 (8)	258 (4)	2.3	-0.518	10, 14	-16
C57	408 (11)	384 (20)**	311 (14)	309 (16)*	262 (13)	0.09	-0.600	10, 0.09	-26
Q	280 (2)	354 (12)	434 (2)	-	-	NS	+0.661	30, 101	+68
O	-	354 (2)	377 (5)	334 (7)	268 (8)	NS	-0.556	40, 119	-39
N	-	370 (4)	298 (17)	316 (19)**	217 (6)	3.6	-0.265	370, 197	-26
OD	507 (3)	440 (6)	455 (8)	361 (10)**	269 (17)	0.09	-0.665	10, 0.09	-51
ND	-	414 (5)	374 (17)	270 (13)	-	1.4	-0.642	10, 0.7	-69
OC	-	-	378 (6)	387 (19)**	319 (19)	2.9	-0.276	350, 151	-30
NC	-	-	318 (15)	309 (13)	292 (10)	NS	-0.051	NS, NS	-13
CN	-	352 (12)	-	315 (4)	281 ^a (6)	NS	-0.210	NS, NS	-4
QD	-	-	405 (11)	273 (5)**	213 ^b (7)	0.5	-0.683	10, 46	-29
QB	-	357 (10)	293 (6)	317 (4)	-	NS	-0.243	NS, NS	-9

* M-W 2-tail p less than 5% between this and adjacent older group.

** M-W 2-tail p less than 2.5% between this and adjacent older group.

^a These animals 19 weeks old.

^b These animals 24 weeks old.

between 12 and 16 weeks. The F_1 's, O and N, betrayed this dichotomy which, in their case, was explicable either in terms of sex chromosome linkage or maternal effect. The fact that OC's display a similar tendency, unlike NC's, to follow the DBA pattern of involution, together with the apparent similarity of CN to NC absolute thymus weight would argue in favour of the latter cause, although analysis of variances in Section 6.4.2 should allow a more definite conclusion to be reached.

It thus appeared that the stimulatory maternal effect that manifested itself with respect to adrenal corticosterone production in animals such as $F_1(0)$ and NC (Chapter 5) might influence thymus involution either, indirectly due to reduced testis function or, directly, due to altered populations of thymic corticosterone receptors.

In order to confirm the existence of strain differences in thymus weight loss, Friedman two-way analyses of variance by ranks were performed on age group means for absolute thymus weight between different combinations of strains. The results of these tests are given in Table 6.8.

Table 6.8 Probabilities (%'s) for Friedman two-way analyses of variance between absolute thymus weight means of different age groups and strains

Strains	Age Periods	p (%)
DBA, CBA, C57	8, 12, 14, 16	6.9
DBA, N, O, C57	10, 12, 14, 16	0.0072
C57, OC, NC, O, N	12, 14, 16	2.5
DBA, OD, ND, O, N	10, 12, 14	7.0
DBA, OD, O	10, 12, 14, 16	4.2
N, OC, NC, C57	12, 14, 16	5.0

The lack of significance in the test of the three parental types was in part due to small strain and age period numbers, but it probably reflected the fact that CBA's differ from DBA and C57 with respect to loss of thymus weight with age; being of similar size to DBA initially, but because of their relatively small degree of involution, becoming heavier than DBA by 16 weeks.

The significant difference apparent in the comparison of DBA and C57 and all their hybrid strains appeared to be due to the fact that C57, OC and NC were more dissimilar to O and N than were DBA, OD and ND. This would suggest the DBA type of involution to be partially dominant, although the results may reflect differences due to the age periods sampled.

6.4.1.2 Thymus dry weight differences between strains within age groups

In order to obtain more exact information concerning thymus dry weight differences, age groups were analysed independently of one another using K-W one-way analysis of variance, to examine major related groups of strains, and M-W 2-tail tests, to investigate differences between pairs within such groups. Tables 6.9 and 6.10 give the probabilities of these tests. Asterisks are used to denote 8 week age group comparisons and M-W results are only tabulated when either an absolute or a relative thymus dry weight comparison yielded a significant result. Probabilities greater than 5% are included to provide some information concerning convergence or divergence.

Absolute dry weights were found to be markedly more significantly different than relative weights. This was explicable, at least in part, by the fact that DBA's increased in body weight from 25.9 gms to

Table 6.9 Kruskal-Wallis one-way analysis of variance of absolute and relative thymus dry weights for combinations of strains for each age group.

Age (Weeks): Strains	All Probabilities Expressed as %'s							
	Absolute Thymus Weights				Relative Thymus Weights			
	10	12	14	16	10	12	14	16
DBA, CBA, C57	0.05*	NS	0.3	0.1	4.0*	NS	NS	6.1
DBA, Q, CBA	NS*	NS	-	-	6.9*	NS	-	-
DBA, N, O, C57	NS	2.6	1.9	0.1	NS	NS	NS	NS
DBA, OD, O	NS	0.7	1.2	0.2	NS	5.9	NS	7.1
DBA, ND, N	NS	8.2	8.4	-	NS	NS	NS	-
DBA, OD, ND, O, N	NS	0.7	6.1	-	NS	2.7	NS	8.5
O, OC, C57	-	1.9	0.3	4.9	-	4.8	1.6	3.2
N, NC, C57	-	NS	NS	2.0	-	NS	NS	NS
O, N, OC, NC, C57	-	3.9	0.2	0.4	-	NS	1.2	1.7
OC, NC, CN	-	-	1.7	-	-	-	0.9	-
N, CN, C57	NS	-	NS	-	NS	-	NS	-
DBA, QD, Q	-	4.8	-	-	-	NS	-	-
Q, QB, CBA	-	9.4	-	-	-	9.0	-	-

* These comparisons were from the 8 weeks age group

Table 6.10 Mann-Whitney two-tailed probabilities of tests between pairs of strains within each age group.

Age (Weeks): Tests	All Probabilities Expressed as %'s							
	Absolute Thymus Weights				Relative Thymus Weights			
	10	12	14	16	10	12	14	16
DBA v. C57	3.5	NS	0.05	0.06	3.17*	NS	NS	1.95
DBA v. CBA	NS*	NS	NS	NS	NS*	NS	NS	NS
CBA v. C57	0.01*	3.39	3.82	0.16	4.7*	NS	NS	NS
C57 v. O	NS	0.72	NS	NS	NS	1.94	NS	NS
C57 v. N	NS	NS	NS	0.47	NS	NS	NS	8.74
DBA v. O	NS	0.93	4.5	0.19	NS	NS	NS	NS
DBA v. N	NS	NS	3.5	NS	NS	NS	NS	NS
DBA v. Q	NS	8.6	-	-	NS	NS	-	-
CBA v. Q	NS*	9.5	-	-	1.47*	NS	-	-
OD v. DBA	NS	1.12	0.34	0.36	4.84*	2.5	NS	1.8
ND v. DBA	NS	3.83	NS	-	NS	NS	NS	-
OC v. C57	-	7.6	0.04	1.58	-	NS	0.23	1.25
NC v. N	-	NS	NS	2.25	-	NS	NS	5.59
O v. N	NS	1.14	NS	4.26	NS	4.8	NS	NS
OD v. ND	NS	NS	NS	-	NS	NS	1.80	-
OC v. NC	-	NS	0.45	NS	-	NS	0.22	NS

* These tests were between 8 week old animals.

only 28.3 gms, whereas C57 body weight increased from 29.4 gms to 35 gms, during the 8 - 16 week period. The greater differences observed between C57-like, rather than DBA-like, strains with respect to relative dry weight may be a reflection of the negative correlation of corticosterone productive capacity, R, with body weight, in C57 mice, observed in Section 6.3.

One of the most prominent features of both K-W and M-W tests was the increase in divergence (indicated by lower probabilities) between strains with age, although significant differences were found between C57 and DBA, and C57 and CBA, at 8 weeks. It was notable, however, that the only significant difference observed in either test, with respect to the absolute or relative thymus dry weights of DBA, CBA and their hybrids, was that observed between DBA, QD and Q at 12 weeks. A Mann-Whitney test between gross mean absolute weights for all ages, for DBA and CBA (using balanced numbers from different age groups), indicated a lack of significant difference between the two strains. The significant result incorporating the backcross may have been real, and not a random occurrence, as different sets of co-adapted genes could still result in identical overall phenotypes in different strains.

Many of the observations made in the previous section with regard to involution were confirmed by the K-W tests that indicated that whereas DBA, OD, ND, N and O were significantly different from each other at only 12 weeks, C57, OC, NC, N and O differed at 12, 14 and 16 weeks, largely due to differences between OC and C57.

When weight comparisons were considered independently of age it became apparent that the absolute thymus weight of DBA was exceptionally low; ND's and, particular, N's being the only strains (apart from CBA and its hybrids) with similar weights.

A maternal influence, possibly operating through suppressed adrenal function (Chapter 5), resulted in OD's and OC's having heavier thymuses than their reciprocal counterparts. This pattern was not followed by N's and O's, however, which differed in the opposite direction, possibly due to a sex chromosome linked factor.

In summary, DBA and C57 were found to be different both with respect to general thymus size and also involution characteristics. DBA and CBA thymuses were statistically indistinguishable although evidence existed that CBA's involuted less markedly than DBA's. The instability of thymus weight between 12 and 16 weeks appeared to be inherited with slight DBA-dominance. On the other hand, thymus size was markedly less in DBA compared with all strains except N and ND, and inheritance of this characteristic whether absolute or relative weights were used, appeared to be C57-dominant.

6.4.2 Comparisons of thymus dry weight variances

Comparison of variances of thymus weights was approached mindful of the limitations on interpretation outlined in Section 5.8.1.1, in connection with the lack of normality of sample distributions of R value, which was likely to reflect the theoretical population distribution.

In the case of thymus weight, variance generated by involution had to be removed if evidence of genetic segregation was to be sought in thymus size, per se. This was enabled by subtracting age group mean weights from the individual values comprising the group so that variance estimates for each group were centred, roughly, on zero. Variances were then added by totalling their products with their degrees of freedom and dividing by the total degrees of freedom to yield a zero-centred estimate for all age groups. Tables 6.11 and 6.12 give, in addition to individual age group variances, estimates of

Table 6.11 Absolute thymus dry weight variances ($\times 10^2$) for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	161 (12)	649 (10)	926 (19)	254 (12)	337 (15)	475 (68)	611 (68)
CBA	118 (15)	-	269 (5)	401 (8)	107 (4)	189 (32)	202 (32)
C57	557 (11)	481 (20)	323 (14)	392 (16)	142 (13)	363 (74)	456 (74)
Q	3 (2)	519 (12)	13 (2)	-	-	381 (16)	508 (16)
O	-	820 (2)	218 (5)	1043 (7)	699 (8)	612 (22)	768 (22)
N	-	622 (4)	691 (17)	919 (19)	251 (6)	680 (46)	783 (46)
OD	1356 (3)	1535 (6)	781 (8)	1195 (10)	267 (17)	718 (44)	1018 (44)
ND	-	1564 (5)	1751 (17)	605 (13)	-	1222 (35)	1398 (35)
OC	-	-	1836 (6)	612 (19)	531 (19)	692 (44)	765 (44)
NC	-	-	1295 (15)	1824 (13)	994 (10)	1324 (38)	1324 (38)
CN	-	372 (12)	-	1664 (4)	2538 (6)	1037 (22)	1047 (22)
QD	-	-	1879 (11)	330 (5)	-	1341 (16)	1614 (16)
QB	-	361 (10)	258 (6)	135 (4)	-	258 (20)	295 (20)

Table 6.12 Relative thymus dry weight variances ($\times 10$) for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	342 (12)	1250 (10)	1506 (19)	429 (12)	391 (15)	781 (68)	1076 (68)
CBA	212 (15)	-	509 (5)	893 (8)	169 (4)	380 (32)	525 (32)
C57	479 (11)	535 (20)	421 (14)	393 (16)	201 (13)	394 (74)	662 (74)
Q	67 (2)	720 (12)	2 (2)	-	-	533 (16)	692 (16)
O	-	654 (2)	214 (5)	1071 (7)	607 (8)	580 (22)	780 (22)
N	-	780 (4)	604 (17)	863 (19)	212 (6)	635 (46)	781 (46)
OD	2679 (3)	2332 (6)	1492 (8)	1136 (10)	288 (17)	984 (44)	1718 (44)
ND	-	1671 (5)	1884 (17)	502 (13)	-	1260 (35)	1587 (35)
OC	-	-	1435 (6)	611 (19)	380 (19)	582 (44)	690 (44)
NC	-	-	1371 (15)	2245 (13)	732 (10)	1425 (38)	1435 (38)
CN	-	473 (12)	-	1571 (4)	1740 (6)	886 (22)	985 (22)
QD	-	-	3587 (11)	319 (5)	-	2477 (16)	2871 (16)
QB	-	553 (10)	444 (6)	274 (4)	-	422 (20)	507 (20)

total variance for absolute and relative thymus weights. Total variance A's were computed in the manner just described, and total variance B's were estimates achieved without centering to zero. In every case, non-segregant variances were greater when uncentred to zero. This was also true for those backcrosses which have been seen already to undergo DBA-type involution. Backcrosses NC and CN, however, gave virtually identical centred and uncentred estimates on account of their lack of thymus involution.

Table 6.13 lists the probabilities (expressed as %'s) of F tests carried out on ratios of segregant:non-segregant strain variances for absolute and relative dry weights, using centred (A) and uncentred (B) total variance estimates.

Absolute, rather than relative, dry weights appeared to provide the most evidence of increased variance amongst backcross mice, although the differences between centred and uncentred estimates appeared slight. With the exceptions of CN/N and OC/O, all backcrosses between DBA and C57 exhibited increased variance in at least one of the absolute or relative weight tests. The lack of such a difference in QB mice may indicate that QD, which were significantly more varied than DBA, may be segregating for a CBA dominant gene.

Although CN and OC were not significantly more varied than their F_1 parents, N and O, this may have been due to the exceptionally high variances of the F_1 's. As CN variances were intermediate in size to those of OC and NC it was concluded that probably no significant sex-linked effects contributed to segregation in thymus size.

It was concluded that these variance comparisons formed good evidence that genes controlling thymus size segregated in hybrids

Table 6.13 Variance ratios for absolute and relative thymus dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.

Strain Ratio	Absolute Thymus Weight				Relative Thymus Weight			
	Ratio Var.A's	p(%)	Ratio Var.B's	p(%)	Ratio Var.A's	p(%)	Ratio Var.B's	p(%)
OD/DBA	1.51	NS	1.67	5.0	1.26	NS	1.60	5.0
ND/DBA	2.57	0.1	2.29	0.5	1.61	NS	1.47	NS
QD/DBA	2.82	0.5	2.64	0.5	3.17	0.1	2.67	0.5
OD/O	1.17	NS	1.33	NS	1.70	NS	2.20	2.5
ND/N	1.80	5.0	1.79	5.0	1.98	2.5	2.03	2.5
QD/Q	3.52	1.0	3.18	2.5	4.65	0.5	4.15	0.1
OC/C57	1.91	0.5	1.68	2.5	1.48	NS	1.04	NS
NC/C57	3.65	0.1	2.90	0.1	3.62	0.1	2.17	0.5
CN/C57	2.86	0.1	2.30	1.0	2.25	1.0	1.49	NS
QB/CBA	1.37	NS	1.46	NS	1.11	NS	0.97	NS
OC/O	1.13	NS	1.00	NS	1.00	NS	0.88	NS
NC/N	1.95	2.5	1.69	NS	2.24	1.0	1.84	5.0
CN/N	1.53	NS	1.34	NS	1.40	NS	1.26	NS
QB/Q	0.68	NS	0.58	NS	0.79	NS	0.73	NS

between DBA and the other two strains, CBA and C57. Inheritance appeared to be CBA-dominant in the backcross QB which may indicate fewer gene differences exist between this parental strain, rather than C57, and DBA.

6.4.3 Thymus weights and involution trends associated with values selected for hetero- or homo-zygosity at the Idh-1, Mod-1, d, b and a genetic loci.

6.4.3.1 Marker-linked influences on thymic involution.

Tables 6.14 and 6.15 give the mean absolute and relative thymus dry weights for each age group, for the values of backcross strains selected according to Idh-1 type. Other statistics shown in the tables are identical to those referred to already, in earlier tables (6.6 and 6.7) giving means for all values. Patterns of difference were similar for absolute and relative weights. Absolute thymus weights of homozygotes, in backcross ND, and heterozygotes, in backcross OC, appeared to be associated with more marked negative correlations with age, to the extent that in the latter strain, by 16 weeks, heterozygotes had significantly lighter absolute thymus weights than homozygotes; (M-W 2-tail $p=0.0076$). This effect was less marked for relative weights. In the absence, apart from OC, of significant correlations with age in backcrosses to C57, differences in interaction with maternal influence could only be assessed in OD and ND crosses; heterozygotes appearing to possess the most contrasting correlations between the two strains. It was concluded from the non-parametric correlations that the DBA-derived Idh-1 allele was linked to factor(s) that were associated with greater thymus weight loss.

Different Mod-1 types (Tables 6.16 and 6.17) of backcrosses to C57, although showing less significant correlations between absolute, or relative, thymus weight and age than DBA backcrosses, displayed more varied slopes. The reduced correlations apparently associated with the C57-derived Mod-1 allele was thus thought to

Table 6.14 Mean absolute thymus dry weights for different Idh-1 types and age groups

		Absolute Weights (MGMS) x 10 ²				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ⁻²)
Age (Weeks):		10	12	14	16				
Strain	Idh Type								
OD	HOM.	895 (2)	1387 (3)	864 (5)	778 (7)	4.4	-0.504	160, 32	-130
OD	HET.	1205 (4)	1242 (4)	1275 (5)	825 (9)	2.6	-0.613	10, 34	-140
ND	HOM.	1040 (4)	1063 (4)	794 (8)	-	NS	-0.707	10, 65	-145
ND	HET.	1300 (4)	1170 (13)	1011 (5)	-	NS	-0.247	NS, NS	-103
OC	HOM.	-	1543 (2)	1254 (10)	1258 (9)*	NS	-0.066	NS, 395	-124
OC	HET.	-	1125 (4)	1316 (9)	974 (10)*	1.1	-0.379	370, NS	-60
NC	HOM.	-	1045 (9)	726 (5)	1168 (4)	NS	+0.121	NS, NS	-47
NC	HET.	-	966 (6)	1222 (6)	898 (6)	NS	+0.094	NS, NS	+33
CN	HOM.	1134 (4)	1368 (2)	1368 (2)	828 ^a (4)	NS	-0.301	NS, NS	-44
CN	HET.	1011 (8)	-	895 (2)	1495 ^a (2)	NS	+0.062	NS, 427	+61

* M-W 2-tail probability for difference between Idh-1 types of same age = 0.76%.

a These animals 19 weeks old.

Table 6.15 Mean relative thymus dry weights for different Idh-1 types and age groups

		Relative Weights x 10 (mgms/100 gms. bwt x 10)				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Age (Weeks):		10	12	14	16				
Strain	Idh Type								
OD	HOM.	334 (2)	509 (3)	303 (5)	257 (7)	4.6	-0.596	50, 6.0	-66
OD	HET.	493 (4)	459 (4)	447 (5)	279 (9)	1.1	-0.723	10, 0.7	-69
ND	HOM.	405 (4)	322 (4)	251 (8)	-	4.7	-0.792	10, 11	-67
ND	HET.	478 (4)	391 (13)	300 (5)	-	NS	-0.519	70, 170	-62
OC	HOM.	-	452 (2)	375 (10)	344 (9)	NS	-0.253	NS, 108	-47
OC	HET.	-	340 (4)	400 (9)	297 (10)	2.1	-0.351	500, NS	-19
NC	HOM.	-	328 (9)	229 (5)	334 (4)	4.8	-0.044	NS, NS	-25
NC	HET.	-	303 (6)	383 (6)	266 (6)	NS	+0.012	NS, NS	+2
CN	HOM.	378 (4)	-	387 (2)	233 ^a (4)	NS	-0.483	NS, NS	-21
CN	HET.	338 (8)	-	243 (2)	1349 ^a (2)	NS	-0.084	NS, NS	+5

a These animals 19 weeks old.

Table 6.16 Mean absolute thymus dry weights for different Mod-1 types and age groups

		Absolute Weights (MGMS) x 10 ²				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Age (Weeks):		10	12	14	16				
Strain	Mod Type								
OD	HOM.	1380 (1)	1463 (3)	1099 (5)	760 (10)	1.0	-0.684	10, 105	-94
OD	HET.	1046 (5)	1096 (5)	1040 (5)	878 (6)	NS	-0.299	NS, 316	-90
ND	HOM.	1167 (6)	936 (6)	880 (4)	-	NS	-0.530	170, 140	-166
ND	HET.	1180 (2)	1258 (11)	876 (9)	-	4.9	-0.508	80, 220	-162
OC	HOM.	-	1003 (3)*	1289 (14)	1065 (7)	NS	-0.099	NS, NS	+9
OC	HET.	-	1525 (3)*	1266 (5)	1134 (12)	NS	-0.268	NS, 104	-133
NC	HOM.	-	818 (6)	743 (6)	970 (6)	NS	+0.417	42, NS	+47
NC	HET.	-	1143 (9)	1179 (4)	1060 (4)	NS	-0.009	NS, NS	-12
CN	HOM.	1011 (10)	-	940 (3)	-	NS	-0.473	NS, 199	-70
CN	HET.	1258 (2)	-	1705 (1)	-	-	+1.000	- -	+146

* M-W 2-tail probability of difference between Mod-1 types of same age = 5%.

Table 6.17 Mean relative thymus dry weights for different Mod-1 types and age groups

		Relative Weights x 10 (mgms./100 gms. bwt x 10)				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Age (Weeks):		10	12	14	16				
Strain	Mod Type								
OD	HOM.	585 (1)	552 (3)	398 (5)	253 (10)	0.6	-0.801	10, 11	-52
OD	HET.	411 (5)	396 (5)	352 (5)	297 (6)	NS	-0.536	50, 17	-50
ND	HOM.	439 (6)	309 (6)	283 (4)	-	NS	-0.713	10, 20	-80
ND	HET.	448 (2)	410 (11)	264 (9)	-	1.4	-0.636	10, 30	-72
OC	HOM.	-	308 (3)	391 (14)	316 (7)	NS	-0.173	NS, NS	-5
OC	HET.	-	447 (3)	377 (5)	321 (12)	NS	-0.274	NS, 46	-43
NC	HOM.	-	249 (6)	234 (6)	281 (6)	NS	+0.289	NS, NS	+12
NC	HET.	-	364 (9)	382 (4)	311 (4)	NS	-0.167	NS, NS	-18
CN	HOM.	340 (10)	-	256 (3)	-	NS	-0.421	NS, NS	-24
CN	HET.	412 (2)	-	491 (1)	-	-	+1.000	- -	+26

be most penetrant in backcrosses to C57. Although OC homozygotes displayed a smaller slope than heterozygotes, the position appeared to be reversed in CN's, although, as only three heterozygotes were obtained for this strain, two of which were from the ten week age group, this result was probably not comparable. Relative weight differences, were, in general, less marked, probably due to the influence of body weight, referred to already in exaggerating negative correlations.

Evidence of maternal interaction was absent in backcrosses to DBA and, if present in OC's and NC's, seemed to affect the slopes of heterozygotes more than homozygotes.

Mice segregating at the dilute locus, d, showed a little evidence of thymus involution differences (Tables 6.18 and 6.19); the K-W test across age groups being significant for absolute and relative weights

Table 6.18 Mean absolute thymus dry weights for different d types and age groups

Age (Weeks): Strain d type		Absolute Weights (MGMS) $\times 10^2$				K-W Across Ages p (%)	r (NPAR)	p ($\% \times 10^2$) NPAR, PAR	Slope ($\times 10^2$)
		10	12	14	16				
OD	HOM.	1027 (3)	1463 (3)	1036 (7)	791 (12)	2.7	-0.556	10, 165	-74
OD	HET.	1177 (3)	1096 (5)	1032 (6)	839 (5)	NS	-0.466	190, 114	-119
ND	HOM.	1157 (5)	1010 (8)	836 (5)	-	NS	-0.480	220, 111	-178
ND	HET.	1192 (3)	1264 (9)	903 (8)	-	NS	-0.508	110, 320	-147
QD	HOM.	-	995 (5)	690 (3)	830 ^a (3)	NS	-0.372	NS, NS	-12
QD	HET.	-	1300 (6)	1005 (2)	815 ^a (4)	NS	-0.618	160, 409	-74

Table 6.19 Mean relative thymus dry weights for different d types and age groups

Age (Weeks): Strain d type		Relative Weights $\times 10$ (mgms./100 gms. bwt $\times 10$)				K-W Across Ages p (%)	r (NPAR)	p ($\% \times 10^2$) NPAR, PAR	Slope ($\times 10^2$)
		10	12	14	16				
OD	HOM.	436 (3)	552 (3)	359 (7)	264 (12)	0.5	-0.735	10, 6	-46
OD	HET.	444 (3)	396 (5)	355 (6)	281 (5)	NS	-0.659	10, 8	-60
ND	HOM.	435 (5)	338 (8)	263 (5)	-	NS	-0.632	20, 18	-85
ND	HET.	451 (3)	406 (9)	274 (8)	-	2.5	-0.678	10, 40	-68
QD	HOM.	-	298 (5)	234 (3)	222 ^a (3)	NS	-0.284	NS, NS	-8
QD	HET.	-	493 (6)	331 (2)	207 ^a (4)	1.7	-0.866	10, 65	-43

a These animals 19 weeks old.

of OD homozygotes, but not for heterozygotes. The position appeared to be reversed in ND and QD mice with heterozygotes, but not homozygotes, of the latter strain being strongly negatively correlated with age. ND heterozygotes, but not homozygotes, showed a significant K-W test difference, similar to QD's, in their relative thymus weights. The maternal influence appeared to alter the thymus age correlations of animals homozygous for the DBA-derived d allele more than those of heterozygotes. The greater differences between slopes of different d, compared with Mod-1, allotypes in backcrosses OD and ND, may have indicated that a factor from chromosome 9, affecting thymus involution, was located nearer the former locus.

Heterozygotes at the b locus (Tables 6.20 and 6.21) appeared to lack the significant correlation observed in homozygotes in ND absolute thymus weights, although this difference disappeared with relative weights. Such a difference could not have been due to an indirect adrenal effect in ND's as it has been shown (Chapter 5) that homozygosity at the b locus was associated with less, not greater, corticosterone productive capacity.

The fact that the b-linked effect on thymus involution was similar with respect to maternal interaction to that observed for adrenal corticosterone production (penetrance being greater in ND's) suggests that the maternal influence may affect these phenotypes through a common mechanism.

The QD thymus correlations for different b types were not regarded as being significantly different.

Segregants at the non-agouti locus, a, exhibited no marked difference between allotypes (Tables 6.22 and 6.23).

Table 6.20 Mean absolute thymus dry weights for different b types and age groups

		Absolute Weights (MGMS) x 10 ²				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Age (Weeks):		10	12	14	16				
Strain	b type								
OD	HOM.	1027 (3)	1378 (2)	813 (5)*	834 (8)	NS	-0.522	80, 84	-78
OD	HET.	1177 (3)	1186 (6)	1173 (8)*	779 (9)	1.5	-0.523	30, 46	-137
ND	HOM.	1050 (3)	1267 (10)	826 (5)	-	NS	-0.715	10, 140	-235
ND	HET.	1242 (5)	969 (7)	909 (8)	-	NS	-0.339	NS, NS	-91
QD	HOM.	-	1197 (8)	758 (3)	783 (3)	NS	-0.518	290, NS	-61
QD	HET.	-	1067 (3)	903 (2)	850 (4)	NS	-0.761	90, NS	-32

* M-W 2-tail probability for difference in b types of same age = 4.5%

Table 6.21 Mean relative thymus dry weights for different b types and age groups

		Relative Weights x 10 (mgms./100 gms. bwt x 10)				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Age (Weeks):		10	12	14	16				
Strain	b type								
OD	HOM.	436 (3)	539 (2)	285 (5)*	283 (8)	1.8	-0.697	10, 7	-48
OD	HET.	444 (3)	427 (6)	402 (8)*	256 (9)	0.5	-0.635	10, 5	-62
ND	HOM.	395 (3)	413 (10)	261 (5)	-	NS	-0.768	10, 40	-95
ND	HET.	469 (5)	319 (7)	276 (8)	-	3.3	-0.528	80, 40	-56
QD	HOM.	-	417 (8)	260 (3)	202 ^a (3)	NS	-0.663	50, 458	-33
QD	HET.	-	370 (3)	293 (2)	222 ^a (4)	NS	-0.857	20, 22	-23

* M-W 2-tail probability for difference in b types of same age = 4.5%

a These animals 19 weeks old.

Table 6.22 Mean absolute thymus dry weights for different a types and ages

		Absolute Weights (MGMS) x 10 ²				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Age (Weeks):		10	12	14	16				
Strain	a type								
QD	HOM.	-	1115 (6)	805 (3)	810 (1)	NS	-0.558	470, NS	-48
QD	HET.	-	1217 (5)	833 (2)	823 (6)	NS	-0.491	440, NS	-62

Table 6.23 Mean relative thymus dry weights for different a types and ages

		Relative Weights x 10 (mgms./100 gms. bwt x 10)				K-W Across Ages p (%)	r (NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Age (Weeks):		10	12	14	16				
Strain	a type								
QD	HOM.	-	376 (6)	278 (3)	210 (1)	NS	-0.558	470, NS	-27
QD	HET.	-	438 (5)	267 (2)	214 (6)	NS	-0.875	10, 137	-36

6.4.3.2 Associations of marker gene allotypes with differences in thymus weight.

In order to examine the gross effects of marker gene-linked factors on thymus weight, Mann-Whitney tests (two-tailed) were performed between homo- and hetero-zygote dry weights drawn from all age groups. Tables 6.24 and 6.25 give the results of such tests which also included comparisons between the allotypes of pooled reciprocal cross thymus weights.

The DBA-derived Idh-1 allele was significantly linked to small thymus weights in ND, pooled ND with OD, and OC absolute values, with the two former associations also being found using relative weights. The factor(s) linked to the Idh-1 locus seemed to have greater penetrance in DBA, rather than C57, backcrosses.

Conversely, the C57 derived allele of Mod-1 was significantly associated with smaller thymus weight in NC, CN, pooled CN with NC, and pooled OC with CN and NC, for both absolute and relative thymus dry weights. The penetrance of this effect appeared to be limited exclusively to backcrosses to C57.

No effects were observed for allotypes of d, b and a.

In order to examine the interaction of maternal influence with these marker gene-linked factors, values of the same allotype were tested between pairs of reciprocal crosses, by means of M-W 2-tail tests. DBA backcrosses yielded no significant interactions for any of the markers. Highly significant effects were observed, however, in C57 backcrosses. These results for the differences in Idh-1 and Mod-1 allotypes between OC and NC are given in Table 6.26.

Table 6.24 Absolute thymus dry weight means for different marker types of individual and combined strains using values from all ages. (Absolute weights (Mgms) $\times 10^2$)

Strains	Marker Type	Mean All ages	p(%) M-W 2-tail test	Strains	Marker Type	Mean All ages	p(%) M-W 2-tail test
OD	Id, Hom	962 (18)		OC	Id, Hom	1283 (21)	
OD	Id, Het	1072 (22)	NS	OC	Id, Het	1134 (23)	3.75
ND	Id, Hom	922 (16)		NC	Id, Hom	984 (18)	
ND	Id, Het	1157 (22)	3.34	NC	Id, Het	1029 (18)	NS
OD and ND	Id, Hom	943 (34)		CN	Id, Hom	1058 (10)	
OD and ND	Id, Het	1115 (44)	3.17	CN	Id, Het	1073 (12)	NS
OD	Mod, Hom	993 (21)		OC and NC	Id, Hom	1145 (39)	
OD	Mod, Het	1035 (22)	NS	OC and NC	Id, Het	1088 (41)	NS
ND	Mod, Hom	1095 (22)		CN and NC	Id, Hom	1010 (28)	
ND	Mod, Het	1008 (16)	NS	CN and NC	Id, Het	1046 (30)	NS
OD and ND	Mod, Hom	1000 (37)		OC, CN, NC	Id, Hom	1127 (49)	
OD and ND	Mod, Het	1065 (44)	NS	OC, CN, NC	Id, Het	1084 (53)	NS
OD	b, Hom	960 (21)		OC	Mod, Hom	1188 (24)	
OD	b, Het	1040 (26)	NS	OC	Mod, Het	1226 (20)	NS
ND	b, Hom	1108 (18)		NC	Mod, Hom	844 (18)	
ND	b, Het	1014 (20)	NS	NC	Mod, Het	1132 (17)	2.24
OD and ND	b, Hom	1028 (39)		CN	Mod, Hom	995 (13)	
OD and ND	b, Het	1028 (46)	NS	CN	Mod, Het	1407 (3)	0.71
OD	d, Hom	971 (27)		OC and NC	Mod, Hom	1040 (42)	
OD	d, Het	1049 (20)	NS	OC and NC	Mod, Het	1183 (37)	NS
ND	d, Hom	1003 (18)		CN and NC	Mod, Hom	907 (31)	
ND	d, Het	1109 (20)	NS	CN and NC	Mod, Het	1173 (20)	0.97
OD and ND	d, Hom	983 (45)		OC, CN, NC	Mod, Hom	1030 (55)	
OD and ND	d, Het	1079 (40)	NS	OC, CN, NC	Mod, Het	1199 (40)	1.08
QD	b, Hom	1014 (14)		QD	a, Hom	992 (10)	
QD	b, Het	934 (9)	NS	QD	a, Het	976 (13)	NS
QD	d, Hom	867 (11)					
QD	d, Het	1089 (12)	NS				

Table 6.25 Relative thymus dry weight means for different marker types of individual and combined strains using values from all ages. (Relative weights x 10. Mgms/100 gms bwt. x 10)

Strains	Marker Type	Mean All ages	p(%) M-W 2-tail test	Strains	Marker Type	Mean All Ages	p(%) M-W 2-tail test
OD	Id, Hom	3435 (18)		OC	Id, Hom	3692 (21)	
OD	Id, Het	3890 (22)	NS	OC	Id, Het	3447 (23)	NS
ND	Id, Hom	3072 (16)		NC	Id, Hom	3016 (18)	
ND	Id, Het	3859 (22)	3.09	NC	Id, Het	3174 (18)	NS
OD and ND	Id, Hom	3264 (34)		CN	Id, Hom	3219 (10)	
OD and ND	Id, Het	3875 (44)	1.62	CN	Id, Het	3285 (12)	NS
OD	Mod, Hom	3618 (21)		OC and NC	Id, Hom	3380 (39)	
OD	Mod, Het	3754 (22)	NS	OC and NC	Id, Het	3327 (41)	NS
ND	Mod, Hom	3512 (16)		CN and NC	Id, Hom	3089 (28)	
ND	Mod, Het	3539 (22)	NS	CN and NC	Id, Het	3219 (30)	NS
OD and ND	Mod, Hom	3573 (37)		OC,CN,NC	Id, Hom	3347 (49)	
OD and ND	Mod, Het	3646 (44)	NS	OC,CN,NC	Id, Het	3318 (53)	NS
OD	b, Hom	3616 (21)		OC	Mod, Hom	3586 (24)	
OD	b, Het	3620 (26)	NS	OC	Mod, Het	3538 (20)	NS
ND	b, Hom	3677 (18)		NC	Mod, Hom	2547 (18)	
ND	b, Het	3393 (20)	NS	NC	Mod, Het	3560 (17)	1.14
OD and ND	b, Hom	3644 (39)		CN	Mod, Hom	3204 (13)	
OD and ND	b, Het	3521 (46)	NS	CN	Mod, Het	4379 (3)	2.50
OD	d, Hom	3512 (27)		OC and NC	Mod, Hom	3141 (42)	
OD	d, Het	3762 (20)	NS	OC and NC	Mod, Het	3548 (37)	NS
ND	d, Hom	3443 (18)		CN and NC	Mod, Hom	2823 (31)	
ND	d, Het	3603 (20)	NS	CN and NC	Mod, Het	3683 (20)	2.06
OD and ND	d, Hom	3485 (45)		OC,CN,NC	Mod, Hom	3156 (55)	
OD and ND	d, Het	3683 (40)	NS	OC,CN,NC	Mod, Het	3610 (40)	5.09
QD	b, Hom	3375 (14)		QD	a, Hom	3301 (10)	
QD	b, Het	2870 (9)	NS	QD	a, Het	3083 (13)	NS
QD	d, Hom	2598 (11)					
QD	d, Het	3709 (12)	NS				

Table 6.26 Mann-Whitney two-tailed probabilities for tests between NC and OC thymus weights of the same marker gene allotype.

Common allotype	Absolute weights M-W 2-tail p (%)	Relative weights M-W 2-tail p (%)
<u>Mod-1</u> heterozygote	NS	NS
<u>Mod-1</u> homozygote	0.01	0.009
<u>Idh-1</u> heterozygote	NS	NS
<u>Idh-1</u> homozygote	0.19	0.74

In summary, major effects on thymus size were exerted by factors linked to the Idh-1 and Mod-1 loci; their penetrances appeared to differ, in that their opposing influences were manifest, to a significant extent, in backcrosses to different parents.

These loci, as well as d and b, probably exert some effect over thymic involution; Mod-1 appearing to exert the strongest effect. This may indicate that the Idh-1-linked factor may control the actual size of the thymus (e.g. the number of reticular cells) whereas the other factors, especially Mod-1, may influence the process of weight loss, rather than size, and would thus be more likely to control qualitative and numerical aspects of thymus receptors to thymocytolytic steroids.

Strong interactions between thymic Idh-1 and Mod-1 allotypes and presumed maternal effects were particularly marked in backcrosses to C57.

6.5 STRAIN DIFFERENCES IN SPLEEN WEIGHT

In the following sections, which examine spleen dry weight differences between strains in an identical fashion to that used for thymus weight, only spleen weights obtained under normal drying conditions will be used, in statistical analyses forming the basis of the conclusions. In certain circumstances, where, for example, data is lacking in certain age groups, spleen weights obtained at a higher drying temperature that were corrected by the regression method, described in Section 6.2.4, will be used to add weight to pre-existing conclusions.

The sub-sections that follow are similar to those presented for the thymus data although the spleen does not undergo such marked involution as the thymus, making differences in growth characteristics with age less obvious.

6.5.1 Strain differences in spleen dry weight within, and across, 14-day age groups.

6.5.1.1 Differences in spleen growth between strains.

The mean, absolute and relative, dry spleen weights are given for each strain, for the 14-day age groups referred to previously, in Tables 6.27 and 6.28. The statistical tests and abbreviations are identical to those used before; the probability of a K-W test (expressed as a percentage) comparing different age groups being followed by the non-parametric correlation coefficient of spleen weight with age (to the nearest day), $r(\text{NPAR})$. The probabilities of this coefficient and its parametric counterpart are then given as percentages multiplied by 10^2 , and finally, an estimate of slope is provided to give some indication of general growth trend.

Table 6.27 Mean absolute spleen dry weights for different strains and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):	8	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p(%x10 ²) NPAR, PAR	Slope (x10 ³)
Strain									
DBA	203 (12)	203 (6)	227 (18)	228 (12)**	171 (8)	1.1	-0.079	NS, NS	-5
CBA	133 (15)	-	132 (5)	136 (8)	139 (4)	NS	+0.397	120, NS	+18
C57	285 (11)**	227 (17)	248 (14)	226 (6)	258 (9)	1.2	-0.205	630, NS	-23
Q	217 (2)	193 (8)	183 (2)	-	-	NS	-0.457	680, 565	-201
O	-	213 (2)	286 (3)	231 (6)	-	NS	-0.241	NS, NS	+18
N	-	198 (4)	221 (17)	205 (17)	175 (2)	NS	-0.230	760, 671	-109
OD	143 (3)	170 (6)*	245 (4)	-	222 (9)	0.9	+0.559	30, 29	+134
ND	-	222 (5)	253 (11)	261 (6)	-	8.0	+0.534	30, 285	+234
OC	-	-	262 (6)	229 (7)*	289 (16)	2.6	+0.228	NS, NS	+77
NC	-	-	245 (15)	234 (9)	221 (10)	NS	-0.224	NS, NS	-70
CN	-	260 (13)	-	267 (4)	282 ^a (6)	NS	+0.100	NS, NS	-3
QD	-	-	197 (11)	223 (5)	176 ^b (7)	NS	-0.122	NS, NS	-35
QB	-	159 (10)	152 (6)	161 (4)	-	NS	-0.153	NS, NS	-1

Table 6.28 Mean relative spleen dry weights for different strains and age groups
(Relative Weights x 10 (mgms/100gms bwt. x 10)).

Age (Weeks):	8	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p(%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain									
DBA	783 (12)	787 (6)	862 (18)	866 (12)**	613 (8)	0.2	-0.233	420, NS	-10
CBA	598 (15)	-	519 (5)	543 (8)	508 (4)	NS	-0.188	NS, 321	-12
C57	970 (11)**	781 (17)	771 (14)	748 (6)	737 (9)	0.1	-0.508	10, 1.2	-31
Q	755 (2)	708 (8)	650 (2)	-	-	NS	-0.558	300, 796	-59
O	-	659 (2)	862 (3)	655 (6)	-	NS	-0.382	NS, NS	-23
N	-	707 (4)	729 (17)	692 (17)	550 (2)	NS	-0.323	210, 138	-50
OD	603 (3)	677 (6)*	907 (4)	-	761 (9)	1.7	+0.375	430, 891	+22
ND	-	799 (5)	848 (11)	793 (6)	-	NS	+0.123	NS, NS	+6
OC	-	-	789 (6)	671 (7)*	825 (16)	4.4	+0.206	NS, NS	+10
NC	-	-	759 (15)	739 (9)*	646 (10)	2.4	-0.388	120, 514	-37
CN	-	857 (13)	-	737 (4)	754 ^a (6)	NS	-0.162	NS, 584	-22
QD	-	-	704 (11)	746 (5)*	459 ^b (7)	0.1	-0.651	10, 0.4	-40
QB	-	650 (10)	593 (6)	588 (4)	-	5.2	-0.233	NS, NS	+2

* M-W 2-tail p less than 5% between this and adjacent older group.

** M-W 2-tail p less than 1% between this and adjacent older group.

a These animals 19 weeks old.

b These animals 24 weeks old.

Absolute and relative spleen dry weight values yielded similar differences in growth trends between strains, the latter demonstrating stronger negative correlations with age.

Strain C57 appeared to differ from DBA and CBA in possessing a more marked negative association between spleen weight and age. This difference between DBA and C57 appeared to be due, in part, to similar patterns of weight loss to those observed for thymus weight; DBA's losing most weight between 14 and 16 weeks, C57's most dramatic reduction being earlier, between 8 and 10 weeks.

Backcrosses to C57 appeared to have more negative correlations between spleen weight and age than did those to DBA. The ranking of strains according to size of negative slope appeared to correspond to mean adrenal corticosterone productive capacities, (R's), for backcrosses to C57; from OC with positive slope and low R to NC with the most negative slope and high R. It would appear, therefore, that spleen weight is governed, at least in part, by the probable corticosterone concentration it experiences, in vivo.

A positive maternal effect appeared to influence relative spleen weight; C57, N, ND and NC having more negative correlations with age than DBA, O, OD and OC. Although this may have been simply the result of insufficient 16-week data for strains O and ND, it was possible that the effect was real and reflected a similar effect observed already (Section 6.3) in connection with correlation of spleen to body weight. In general, the maternal effect, whether positive or negative, resulted in the offspring of $F_1(N)$ dams possessing more negative associations between relative spleen weight and age.

Although the growth trends of DBA and CBA spleens appeared

similar, it was conceivable that additional values at 10 weeks could have given CBA's a more negative correlation of relative spleen weight with age, perhaps between those of DBA and C57. The F_1 hybrid strain, Q, certainly appeared to resemble the analogous C57 hybrid, $F_1(N)$, in possessing a markedly negative correlation, although backcross QD spleens appeared to lose more weight with age than those of backcross ND. The latter difference may, however, have been spurious due to the exceptionally low spleen weights of the 24-week old QD's.

A Friedman two-way analysis of variance of the absolute and relative spleen dry weight means of DBA, CBA and C57, for the 8-, 12-, 14- and 16-week age groups, gave probabilities of 0.042 and 0.069, respectively. Similar tests carried out on available DBA, N, O and C57 means revealed no significant differences for either absolute or relative weights. Meaningful tests involving other strain combinations were impossible due to the absence of values for certain age groups.

6.5.1.2 Spleen dry weight differences between strains within age groups.

Tables 6.29 and 6.30 give the probabilities of K-W and M-W tests performed on various strain combinations within age groups. To save space, only M-W tests that were significant for one or more, age groups, for either absolute or relative spleen weight, have been listed. As with thymus weight, strain differences appeared to become greater with age supporting the observations concerning different growth patterns contained in the previous section.

Despite the occurrence of a number of significant K-W comparisons, the general conclusion was formed that, compared to thymus weight, there appeared to be relatively trivial differences in spleen size between DBA, C57 and their hybrid strains.

Table 6.29 Kruskal-Wallis one-way analysis of variance of absolute and relative spleen dry weights for combinations of strains for each age group (All probabilities expressed as %'s).

Age (Weeks): Strains	Absolute Spleen Weights				Relative Spleen Weights			
	10	12	14	16	10	12	14	16
DBA, CBA, C57	0.05*	0.05	0.05	0.1	0.05*	0.05	0.05	0.4
DBA, Q, CBA	0.05*	0.1	-	-	0.2*	0.1	-	-
DBA, N, O, C57	NS	5.8	NS	-	NS	5.5	2.9	-
DBA, OD, O	NS	NS	-	-	NS	NS	-	-
DBA, ND, N	NS	NS	NS	-	NS	5.5	3.5	-
DBA, OD, ND, O, N	NS	NS	-	-	NS	6.2	-	-
O, OC, C57	-	NS	NS	-	-	NS	NS	-
N, NC, C57	-	NS	NS	1.9	-	NS	NS	2.0
O, N, OC, NC, C57	-	NS	NS	-	-	NS	NS	-
OC, NC, CN	-	-	6.3	-	-	-	NS	-
N, CN, C57	5.2	-	4.8	-	6.6	-	NS	-
DBA, QD, Q	-	4.1	-	-	-	0.2	-	-
Q, QB, CBA	-	0.9	-	-	-	2.0	-	-

* These comparisons were from the 8 weeks age group.

Table 6.30 Mann-Whitney two-tailed probabilities for tests between pairs of strains within each age group (all probabilities expressed as %'s).

Age (Weeks): Tests	Absolute Spleen Weights				Relative Spleen Weights			
	10	12	14	16	10	12	14	16
DBA v. C57	0.01*	3.36	NS	0.03	0.22*	6.49	NS	2.74
DBA v. CBA	0.009*	0.01	0.009	7.27	0.04*	0.01	0.009	4.85
CBA v. C57	0.009*	0.02	0.07	0.28	0.009*	0.02	0.13	0.28
C57 v. N	NS	NS	NS	3.64	NS	NS	NS	3.64
DBA v. O	NS	7.97	NS	-	NS	NS	1.35	-
DBA v. N	NS	NS	NS	NS	NS	2.24	1.41	NS
DBA v. Q	NS	9.47	-	-	NS	1.05	-	-
CBA v. Q	2.94*	9.52	-	-	8.82*	9.52	-	-
OD v. DBA	0.88*	NS	-	0.55	4.84*	NS	-	1.11
ND v. DBA	8.23	NS	NS	-	NS	NS	NS	-
QD v. DBA	-	3.50	NS	-	-	0.15	NS	-
ND v. N	NS	NS	3.63	-	NS	9.06	NS	-
NC v. C57	-	NS	NS	5.35	-	NS	NS	4.35
CN v. N	4.45	-	1.77	-	7.90	-	NS	-
OC v. NC	-	NS	NS	0.18	-	NS	7.12	0.03
OD v. ND	3.03	NS	-	-	8.23	NS	-	-
QB v. CBA	-	0.87	2.83	-	-	1.73	NS	-
QB v. Q	0.85	7.14	-	-	NS	NS	-	-

* These tests were between 8 week old animals.

On the other hand, tests involving CBA strain, and its hybrids, suggested that this strain was significantly different from DBA (and C57) using either absolute or relative spleen weights. This appeared to be a CBA-recessive character as the backcross QB was the only hybrid possessing spleens of sizes approaching those of CBA. Only comparison of variances would enable a conclusion to be drawn as to whether spleen size in strain CBA was a genetic characteristic intrinsic to the organ itself, or whether it was merely a reflection of a markedly different hormonal environment. It was probable that the latter was the cause of the relatively minor differences between DBA and C57 spleen weights.

6.5.2 Comparisons of spleen dry weight variances.

Tables 6.31 and 6.32 contain the variances of spleen absolute and relative weights for each strain and age group. Also included are the two estimates of total variance referred to in Section 6.4.2; estimate A being the zero-centred estimate.

Table 6.33 gives the variance ratios and probabilities for absolute and relative spleen weights. Only two strains, OC and CN, gave significant probabilities at the 5% level, and these were obtained in only one out of the four comparisons for each strain. Even when variance estimates (not shown in the table) were used for pooled segregant and non-segregant lines, no significant effects could be observed.

It was thus concluded that, from the data available, there existed little evidence of genetic differences directly affecting spleen size between the three parental lines. The differences in spleen size that did exist, particularly in strain CBA and its hybrids, were, therefore, thought to reflect, indirectly, differences in function or influence of

Table 6.31 Absolute spleen dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	129 (12)	67 (6)	168 (18)	257 (12)	110 (8)	149 (56)	189 (56)
CBA	59 (15)	-	10 (5)	23 (8)	14 (4)	34 (32)	35 (32)
C57	175 (11)	164 (17)	86 (14)	170 (6)	170 (9)	138 (57)	185 (57)
Q	201 (2)	116 (8)	4 (2)	-	-	92 (12)	104 (12)
O	-	51 (2)	233 (3)	274 (6)	-	189 (11)	271 (11)
N	-	137 (4)	185 (17)	177 (17)	7 (2)	159 (40)	173 (40)
OD	63 (3)	107 (6)	159 (4)	174 (3)	65 (9)	84 (25)	218 (25)
ND	-	123 (8)	242 (11)	293 (6)	-	197 (25)	253 (25)
OC	-	-	151 (6)	211 (7)	252 (16)	207 (29)	272 (29)
NC	-	-	329 (15)	58 (9)	115 (10)	185 (34)	195 (34)
CN	-	206 (13)	-	34 (4)	485 ^a (6)	227 (23)	237 (23)
QD	-	-	117 (11)	222 (5)	45 ^b (7)	106 (23)	134 (23)
QB	-	25 (10)	11 (6)	26 (4)	-	19 (20)	20 (20)

Table 6.32 Relative spleen dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group means.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	145 (12)	129 (6)	289 (18)	399 (12)	84 (8)	221 (56)	294 (56)
CBA	99 (15)	-	13 (5)	31 (8)	8 (4)	54 (32)	68 (32)
C57	145 (11)	117 (17)	77 (14)	73 (6)	100 (9)	98 (57)	167 (57)
Q	104 (2)	142 (8)	1 (2)	-	-	100 (12)	110 (12)
O	-	31 (2)	120 (3)	213 (6)	-	134 (11)	227 (11)
N	-	142 (4)	163 (17)	194 (17)	16 (2)	158 (40)	173 (40)
OD	74 (3)	123 (6)	169 (4)	170 (3)	119 (9)	107 (25)	226 (25)
ND	-	102 (8)	239 (11)	110 (6)	-	153 (25)	164 (25)
OC	-	-	180 (6)	285 (7)	153 (16)	175 (29)	217 (29)
NC	-	-	235 (15)	95 (9)	54 (10)	137 (34)	162 (34)
CN	-	176 (13)	-	52 (4)	348 ^a (6)	182 (23)	213 (23)
QD	-	-	150 (11)	202 (5)	33 ^b (7)	114 (23)	264 (23)
QE	-	36 (10)	26 (6)	34 (4)	-	29 (20)	38 (20)

a These animals 19 weeks old.

b These animals 24 weeks old.

Table 6.33 Variance ratios for absolute and relative spleen dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.

Strain Ratio	Absolute spleen weight				Relative spleen weight			
	Ratio Var.A's	p(%)	Ratio Var.B's	p(%)	Ratio Var.A's	p(%)	Ratio Var.B's	p(%)
OD/DBA	0.56	NS	1.15	NS	0.48	NS	0.77	NS
ND/DBA	1.32	NS	1.34	NS	0.69	NS	0.56	NS
QD/DBA	0.71	NS	0.71	NS	0.52	NS	0.90	NS
OD/O	0.44	NS	0.80	NS	0.80	NS	1.00	NS
ND/N	1.24	NS	1.46	NS	0.97	NS	0.95	NS
QD/Q	1.15	NS	1.29	NS	1.14	NS	2.40	NS
OC/C57	1.50	NS	1.47	NS	1.79	5.0	1.30	NS
NC/C57	1.34	NS	1.05	NS	1.40	NS	0.97	NS
CN/C57	1.64	NS	1.28	NS	1.86	5.0	1.28	NS
QB/CBA	0.56	NS	0.57	NS	0.54	NS	0.56	NS
OC/O	1.10	NS	1.00	NS	1.31	NS	0.96	NS
NC/N	1.16	NS	1.13	NS	0.87	NS	0.94	NS
CN/N	1.43	NS	1.37	NS	1.15	NS	1.23	NS
QB/Q	0.21	NS	0.19	NS	0.29	NS	0.35	NS

organs such as the testis and adrenal. The Y-linked difference in testis size mentioned in Section 6.1, was thought to be a possible cause of the greatly reduced spleen weights of strain CBA.

6.5.3 Spleen weights and growth trends associated with values selected for hetero- or homo-zygosity at the Idh-1, Mod-1 d, b and a genetic loci.

6.5.3.1 Marker-linked influences on splenic weight change.

Tables 6.34 and 6.35 give the K-W and correlation statistics for absolute and relative spleen dry weights selected for different Idh-1 type. No important differences were observed between absolute and relative weights although the difference between allotypes in OD's appeared to be greater in terms of slope in the case of the latter weights. The most significant differences between allotypes in growth trends were apparent in backcrosses to C57, in which the DBA-derived Idh-1 allele was linked to reduced spleen weight increase with age. A presumed maternal effect which, in general terms, resulted in greater spleen accretion with age in OC's than in NC's, did not appear to interact, appreciably, with Idh-1 type. DBA backcrosses, however, although displaying reduced expression of the marker-linked effect, betrayed signs of an Idh-1 type/maternal influence interaction in an apparent reversal of allotype-specific correlations in the reciprocal crosses.

Considering spleens selected for different Mod-1 type, absolute and relative weights, as shown in Tables 6.36 and 6.37, both demonstrated marked differences in their correlations with age in backcrosses to DBA. Interaction of maternal influence with Mod-1 type, as with Idh-1, seemed to result in reversal of differences between allotypes in OD's and ND's; the DBA-derived allele being the most affected. It appeared from OC's and NC's, where expression of the Mod-1-linked factor was less confused by maternal interaction, that the C57-derived allele was linked to greater splenic weight decrease with age.

Table 6.34 Mean absolute dry spleen weights for different Idh-1 types and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Id Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ⁵)
OD	Hom.	181 (2)	-	145 (2)	227 (4)	NS	+0.553	610, 752	+132
OD	Het.	165 (4)	236 (3)	189 (1)	219 (5)	NS	+0.460	570, NS	+101
ND	Hom.	218 (4)	210 (1)	262 (4)	-	NS	+0.245	NS, NS	+204
ND	Het.	196 (4)	257 (10)	260 (2)	-	4.0	+0.740	10, 666	+263
OC	Hom.	-	255 (2)	218 (4)	304 (8)	2.1	+0.469	450, NS	+161
OC	Het.	-	266 (4)	242 (3)	274 (8)	NS	-0.029	NS, NS	+11
NC	Hom.	-	235 (9)	213 (4)*	242 (4)	NS	+0.075	NS, NS	+1
NC	Het.	-	259 (6)	251 (5)*	206 (6)	3.1	-0.514	170, 249	-178
CN	Hom.	271 (4)	-	282 (2)	311 ^a (4)	NS	+0.320	NS, NS	+50
CN	Het.	255 (9)	-	252 (2)	222 ^a (2)	NS	-0.162	NS, NS	-50

Table 6.35 Mean relative dry spleen weights for different Idh-1 types and age groups
(Relative Weights x 10 (mgms./100 gms bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Id Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ⁴)
OD	Hom.	667 (2)	-	489 (2)	786 (4)	NS	+0.485	NS, NS	+25
OD	Het.	682 (4)	872 (3)	696 (1)	742 (5)	NS	+0.127	NS, NS	-2
ND	Hom.	831 (4)	657 (1)	806 (4)	-	NS	-0.059	NS, NS	-9
ND	Het.	718 (4)	868 (10)	769 (2)	-	NS	+0.303	NS, NS	+21
OC	Hom.	-	750 (2)	625 (4)	813 (8)	2.1	+0.489	380, NS	+23
OC	Het.	-	808 (4)	732 (3)	838 (8)	NS	+0.078	NS, NS	+6
NC	Hom.	-	731 (9)	671 (4)*	697 (4)	NS	-0.097	NS, NS	-19
NC	Het.	-	803 (6)	794 (5)*	611 (6)	0.8	-0.571	80, 165	-65
CN	Hom.	891 (4)	-	793 (2)	852 ^a (4)	NS	-0.1129	NS, NS	-5
CN	Het.	842 (9)	-	681 (2)	558 ^a (2)	6.4	-0.349	NS, 377	-36

* M-W 2-tail probability of difference between Id types of the same age = 3.17%.

^a These animals 19 weeks old.

Table 6.36 Mean absolute dry spleen weights for different Mod-1 types and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):			10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain	Mod	Type								
OD	Hom.		141 (1)	-	189 (1)	220 (6)	7.6	+0.793	30, 15	+158
OD	Het.		176 (5)	245 (4)	145 (2)	228 (3)	7.4	+0.360	940, NS	+94
ND	Hom.		220 (6)	236 (4)	203 (2)	-	NS	+0.101	NS, NS	-73
ND	Het.		168 (2)	262 (7)	290 (4)	-	5.0	+0.667	60, 476	+361
OC	Hom.		-	278 (3)	203 (3)	293 (6)	4.1	+0.271	NS, NS	-23
OC	Het.		-	246 (3)	248 (4)	287 (10)	NS	+0.212	NS, NS	+124
NC	Hom.		-	255 (6)	230 (5)	229 (6)	NS	-0.289	NS, 917	-118
NC	Het.		-	238 (9)	240 (3)	208 (4)	NS	-0.136	NS, NS	-50
CN	Hom.		255 (11)	-	264 ^a (3)	-	NS	+0.422	670, NS	+32
CN	Het.		283 (2)	-	278 ^a (1)	-	-	-0.50	- -	-27

Table 6.37 Mean relative dry spleen weights for different Mod-1 types and age groups
(Relative Weights x 10 (mgms/100 gms bwt. x 10)).

Age (Weeks):			10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain	Mod	Type								
OD	Hom.		595 (1)	-	696 (1)	764 (6)	NS	+0.724	90, 232	+34
OD	Het.		694 (5)	907 (4)	489 (2)	757 (3)	7.2	+0.086	NS, NS	-2
ND	Hom.		824 (6)	793 (4)	702 (2)	-	6.7	-0.554	310, 36	-59
ND	Het.		628 (2)	880 (7)	839 (4)	-	NS	+0.383	980, NS	+38
OC	Hom.		-	854 (3)	589 (3)	871 (6)	4.4	+0.275	NS, NS	-16
OC	Het.		-	723 (3)	732 (4)	798 (10)	NS	+0.147	NS, NS	+21
NC	Hom.		-	776 (6)	718 (5)	661 (6)	7.3	-0.541	120, 180	-44
NC	Het.		-	748 (9)	792 (3)	623 (4)	NS	-0.256	NS, NS	-33
CN	Hom.		843 (11)	-	716 ^a (3)	-	NS	+0.331	NS, NS	+11
CN	Het.		932 (2)	-	799 ^a (1)	-	-	-1.00	- -	-47

^a These animals 19 weeks old.

Table 6.38 Mean absolute spleen dry weights for different d types and age groups (Absolute Weights (MGMS.) x 10).

Age (Weeks):	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Strain d Type								
OD Hom.	164 (3)	-	189 (1)	225 (7)	5.2	+0.747	20, 6	+165
OD Het.	176 (3)	245 (4)	145 (2)	212 (2)	NS	+0.240	NS, NS	+50
ND Hom.	219 (5)	252 ^a (6)	239 (3)	-	NS	+0.183	NS, NS	+107
ND Het.	187 (3)	254 (5)	284 (3)	-	5.6	+0.785	20, 234	+335
QD Hom.	-	201 (5)	213 (3)	174 ^a (3)	NS	-0.228	NS, NS	-38
QD Het.	-	193 (6)	237 (2)	178 ^a (4)	NS	-0.119	NS, NS	-34

Table 6.39 Mean relative dry spleen weights for different d types and age groups (Relative Weights x 10 (mgms./100gms. bwt. x 10)).

Age (Weeks):	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain d Type								
OD Hom.	697 (3)	-	696 (1)	773 (7)	NS	+0.599	150, 233	+30
OD Het.	657 (3)	907 (4)	489 (2)	720 (2)	NS	-0.057	NS, NS	-9
ND Hom.	820 (5)	851 (6)	756 (3)	-	NS	-0.169	NS, NS	-25
ND Het.	700 (3)	845 (5)	831 (3)	-	NS	+0.263	NS, NS	+35
QD Hom.	-	670 (5)	721 (3)	464 ^a (3)	4.7	-0.530	470, 75	-34
QD Het.	-	732 (6)	782 (2)	455 ^a (4)	2.4	-0.722	40, 26	-45

^a These animals 24 weeks old.

Values differing at the d locus, shown in Tables 6.38 and 6.39, showed similar patterns of difference to those observed for Mod-1, except the correlations of different d types in ND's were less pronounced using relative weights. When sample sizes in this strain were augmented, however, by the inclusion of 16-week corrected spleen weights, correlations became more significant; correlations, probabilities (parametric and non-parametric, expressed as %'s x 10²), and slopes of relative spleen weights were -0.444, 430, 274, -51 for homozygotes, and +0.153, NS, NS, +22 for heterozygotes. It would seem that CBA and DBA

do not differ with respect to d- and Mod-1-linked splenic weight change with age as correlations, whether using absolute or relative weights, did not reveal significant differences, in backcross QD.

The b locus, when homozygous for the DBA allele (Tables 6.40 and 6.41), appeared to be linked to greater spleen weight increase with age, in both absolute and relative weights, in the backcrosses, OD and ND. Increasing the ND sample size by consideration of 16-week corrected relative spleen values, as before, resulted in the following increased differences in correlation statistics; -0.194, NS, NS, -8 for heterozygotes, and +0.522, 340, 140, +94 for homozygotes. As in the case of d, QD spleens showed no difference in effect of b allotype on weight correlation with age.

Finally, from the statistics for spleen weights associated with different a types, shown in Tables 6.42 and 6.43, it was concluded that some factor, linked to this locus, may reduce splenic weight loss when homozygous for the DBA allele.

Identical results for all markers were achieved with ND correlations when corrected 16-week spleens were included, and in many cases, especially in those referred to already, significances were increased.

6.5.3.2 Associations of marker gene allotypes with differences in spleen weight

The gross effects of marker gene-linked factors on spleen dry weights were evaluated, as for thymus, by performing Mann-Whitney two-tailed tests between values of different allotype, for individual and pooled backcrosses. Table 6.44 gives the results of such tests, some of which have been indicated already, by means of asterisks in the growth trend tables (6.34 - 6.41).

Table 6.40 Mean absolute uncorrected spleen dry weights for different b types and age groups (Absolute Weights (MGMS.) x 10).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Strain	b type								
OD	Hom.	164 (3)	244 (1)	145 (2)	237 (4)	6.4	+0.631	100, 96	+154
OD	Het.	176 (3)	245 (3)	189 (1)	211 (5)	NS	+0.211	NS, NS	+27
ND	Hom.	219 (3)	280 (6)*	286 (3)	-	NS	+0.712	50, 101	+561
ND	Het.	199 (5)	220 (5)*	236 (3)	-	NS	+0.452	NS, NS	+143
QD	Hom.	-	202 (8)	190 (3)	171 ^a (3)	NS	-0.199	NS, NS	-40
QD	Het.	-	183 (3)	272 (2)	180 ^a (4)	NS	-0.061	NS, NS	-48

* M-W 2-tail probability for difference between b types of same age = 0.87%.

Table 6.41 Mean relative uncorrected spleen dry weights for different b types and age groups (Relative Weights x 10 (mgms/100 gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain	b type								
OD	Hom.	697 (3)	890 (1)	489 (2)	806 (4)	5.1	+0.425	740, NS	+26
OD	Het.	657 (3)	912 (3)	696 (1)	725 (5)	NS	-0.072	NS, NS	-12
ND	Hom.	826 (3)	926 (6)*	850 (3)	-	NS	+0.382	NS, NS	+49
ND	Het.	744 (5)	755 (5)*	737 (3)	-	NS	-0.017	NS, NS	-3
QD	Hom.	-	731 (8)	657 (3)	442 ^a (3)	3.0	-0.645	60, 12	-43
QD	Het.	-	632 (3)	878 (2)	472 ^a (4)	3.0	-0.691	200, 158	-41

* M-W 2-tail probability for differences between b types of same age = 5.19%.

Table 6.42 Mean absolute uncorrected spleen dry weights for different a types and age groups (Absolute Weights (MGMS.) x 10).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
Strain	a type								
QD	Hom.	-	184 (6)	219 (3)	169 ^a (1)	NS	0.019	NS, NS	-22
QD	Het.	-	211 (5)	229 (2)	178 ^a (6)	NS	-0.306	NS, 629	-59

Table 6.43 Mean relative uncorrected spleen dry weights for different a types and age groups (Relative Weights x 10 (mgms/100 gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
Strain	a type								
QD	Hom.	-	665 (6)	758 (3)	439 ^a (1)	NS	-0.272	NS, 536	-36
QD	Het.	-	750 (5)	728 (2)	462 ^a (6)	1.1	-0.796	10, 4	-48

^a These animals 24 weeks old.

Table 6.44 Mann-Whitney two-tailed tests between mean absolute and relative spleen dry weights selected for different marker allotypes.

Strain(s)	Marker Allotypes	Age(s) (weeks)	Absolute weights		Relative weights	
			Mean	M-W p(%)	Mean	M-W p(%)
NC	Id Hom	14	213 (4)		671 (4)	
NC	Id Het	14	251 (5)	1.59	794 (5)	3.17
OC, NC	Id Hom	14	216 (8)		648 (8)	
OC, NC	Id Het	14	248 (8)	2.07	771 (8)	4.99
NC, CN	Id Hom	16	277 (8)		775 (8)	
NC, CN	Id Het	16	210 (8)	1.04	598 (8)	0.30
OC, NC, CN	Id Hom	16	290 (16)		794 (16)	
OC, NC, CN	Id Het	16	242 (16)	1.52	718 (16)	8.60
ND	b Hom	12	280 (6)		926 (6)	
ND	b Het	12	220 (5)	0.87	755 (5)	5.19
ND	b Hom	ALL	266 (12)		1116 (12)	
ND	b Het	ALL	216 (13)	1.35	747 (13)	0.24

It can be seen from Table 6.44 that although the DBA-derived allele of Idh-1 was found, at 14 weeks, to be linked to higher absolute and relative spleen weights in NC's, alone, and when pooled with OC's, it was found to be associated with lower weight by 16 weeks. These results, as well as the absence of significance when all ages were tested for Idh-1 allotype-associated differences, serve to emphasise the divergent correlations noted for different Idh-1 types, in the last section.

This marker thus appeared to be linked to a factor controlling spleen weight change, rather than one determining general organ size or architecture.

The DBA-derived allele of the b locus, on the other hand, appeared to be consistently linked at all ages, especially 12 weeks, with higher spleen weight. An outside possibility of genetic segregation for a factor controlling spleen size in ND mice was suggested by the finding that, when corrected 16-week values (referred to earlier) were incorporated in the computation of variance estimates, F tests, significant at the 5% level, were obtained for the variance ratios ND/DBA and ND/N. Segregation for this gene might also have accounted for the significant F tests observed for the variance ratios OC/C57 and NC/C57.

To assess maternal influence interactions with marker allotypes, comparisons, using M-W 2-tail tests, between reciprocal strains for values of the same marker type, were made and are given in Table 6.45.

Table 6.45 Mann-Whitney two-tailed probabilities for tests between OD and ND spleen weights of the same marker gene allotype.

Common allotype	Absolute weights M-W 2-tail p(%)	Relative weights M-W 2-tail p(%)
<u>Idh-1</u> heterozygote	3.19	NS
<u>Idh-1</u> homozygote	NS	NS
<u>Mod-1</u> heterozygote	2.53	NS
<u>Mod-1</u> homozygote	8.04	2.06
<u>d</u> heterozygote	NS	NS
<u>d</u> homozygote	4.82	2.2
<u>b</u> heterozygote	NS	NS
<u>b</u> homozygote	0.16	0.37

Although absolute and relative weight results were ambiguous for Mod-1 allotypes, it appeared, from tests of different d types, that factor(s) affecting spleen weight change, on chromosome 9, interact with the maternal influence when homozygous for DBA-derived alleles.

The weak evidence of maternal interaction with the Idh-1-linked factor suggested that the C57-derived allele may be most affected, in this case.

As these tests reflected maternal interaction by assessing gross size differences, it was natural that different b haplotypes should display the most contrasted difference of all markers; the DBA-derived b-linked factor being highly significantly different in its effect on spleen weight in the two backcrosses, while maternal effect interaction with the C57-derived allele appeared negligible.

Summing up, marker gene-linked effects on spleen weight were observed for the Idh-1, Mod-1, d, b and a loci. Maternal interactions with specific allotypes were restricted to backcrosses to DBA. The DBA-derived alleles of the b, Mod-1 and a loci appeared to be linked to increased (or less decreased) spleen weight with age; allotypes of the former coat colour gene seeming to be linked to significant weight differences over all ages, and to contribute, possibly, to increased spleen weight variances in ND's, as the result of gene(s) affecting intrinsic spleen structure.

The Idh-1 locus, in contrast to Mod-1, was linked to gene(s) that, when derived from DBA, reduced spleen weight with age, and were maximally penetrant in C57 backcrosses (unlike Mod-1 which showed greatest effect in DBA backcrosses). Factors linked to all gene markers, except a, segregated in crosses between DBA and C57, whereas the latter was the only locus for which effects on spleen weight were observed in the CBA hybrid line, QD.

6.6 STRAIN DIFFERENCES IN TESTIS WEIGHT

In this section, mean testis dry weight (average weight of pair of organs) will be examined in a similar manner to that used for thymus and spleen weight.

6.6.1 Strain differences in testis dry weight within, and across, 14-day age groups.

6.6.1.1 Differences in testis growth between strains.

Tables 6.46 and 6.47 give the mean absolute and relative testis dry weights for each strain and age group, accompanied by the statistics used in previous organ weight tables.

Considering absolute weight first; the parental strains DBA and C57 both showed significant positive correlations with age; the probabilities of both correlation coefficients (NPAR and PAR), and the K-W analysis of variance result, perhaps indicating a stronger association in the former strain. The general growth trends were of similar slope. Strain CBA, on the other hand, demonstrated a significant negative correlation with age.

Although all F_1 hybrid mice possessed testes with apparently negative growth trends, none of them was justifiable by a significant correlation coefficient.

Among the backcrosses only QD, QB, ND and NC revealed significant relationships with age, although CN testis weight appeared to fluctuate significantly, on the basis of K-W test, without demonstrating any significant overall trend. Backcross QD was notable for the similarity of its correlations and slope to DBA, whereas ND, while possessing a significant positive correlation, appeared to have a smaller slope; possibly indicating the segregation of fewer genes affecting this

Table 6.46 Mean absolute testis dry weights for different strains and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):	8	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x 10 ³)
Strain									
DBA	139 (12)	152 (6)	150 (19)	156 (12)	162 (14)	0.2	+0.521	10, 0.1	+36
CBA	78 (15)	-	81 (**)	60 (**)	82 (4)	0.09	-0.341	280, NS	-11
C57	135 (11)	130 (19)	148 (14)	146 (15)	150 (13)	NS	+0.214	360, 89	+33
Q	131 (2)	131 (9)	119 (2)	-	-	NS	-0.231	NS, NS	-36
O	-	178 (2)	166 (4)	188 (7*)	166 (8)	7.7	-0.271	NS, NS	-35
N	-	150 (4)	166 (17)	156 (19)	155 (6)	NS	-0.194	NS, NS	-27
OD	166 (3*)	141 (6)	143 (8)	157 (10)	155 (17)	8.3	+0.205	910, NS	+12
ND	-	160 (5)	170 (16)	176 (12)	173 (7)	NS	+0.274	440, NS	+24
OC	-	-	187 (6*)	168 (19)	174 (20)	6.1	-0.157	NS, NS	-18
NC	-	-	153 (15)	166 (13)	168 (10)	NS	+0.264	540, 405	+77
CN	-	143 (13*)	-	178 (4)	164 ^a (6)	2.1	+0.118	NS, NS	+14
QD	-	-	139 (10)	140 (5)	159 ^b (7)	NS	+0.407	300, 139	+34
QB	-	79 (10)	74 (6*)	68 (4)	-	5.4	-0.513	100, NS	-4

Table 6.47 Mean relative testis dry weights for different strains and age groups
(Relative Weights x 10 (mgms/100gms bwt. x 10)).

Age (Weeks):	8	10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x 10 ²)
Strain									
DBA	537 (12)	586 (6)	566 (19)	593 (12)	574 (14)	2.6	+0.329	40, 182	+5
CBA	353 (15)	-	320 (**)	240 (8*)	300 (4)	0.09	-0.759	10, 0.1	-16
C57	459 (11)	444 (19)	461 (14)	444 (15)	430 (13)	NS	-0.168	800, NS	-4
Q	460 (2)	482 (9)	422 (2)	-	-	NS	-0.208	NS, NS	-5
O	-	551 (2)	500 (4)	533 (7*)	468 (8)	0.9	-0.626	10, 10	-25
N	-	536 (4)	546 (17)	523 (17)	510 (6)	NS	-0.372	70, 155	-17
OD	701 (3*)	563 (6)	523 (8)	541 (10)	519 (17)	5.5	-0.321	170, 6	-23
ND	-	574 (5)	562 (16)	539 (12)	548 (7)	NS	-0.147	NS, NS	-8
OC	-	-	562 (6*)	506 (19)	504 (20)	4.5	-0.247	510, 975	-12
NC	-	-	490 (15)	507 (13)	492 (10)	NS	+0.106	NS, NS	+7
CN	-	471 (13)	-	492 (4)	436 ^a (6)	NS	-0.417	240, 410	-9
QD	-	-	492 (10)	472 (5)	415 ^b (7)	9.8	-0.413	280, 138	-12
QB	-	321 (10)	291 (6*)	247 (4)	-	0.4	-0.464	200, NS	-1

* M-W 2-tail p less than 5% between this and adjacent older group.

** M-W 2-tail p less than 1% between this and adjacent older group.

a These animals 19 weeks old.

b These animals 24 weeks old.

character in the former backcross. Reciprocal backcrosses with $F_1(N)$ dams seemed to display more positive growth trends of absolute testis with age than those mothered by $F_1(0)$ mice.

Although, in general, correlations with age being more negative, statistics using relative testis weights showed similar differences between strains with respect to growth trends, although the significantly negative correlation of QD relative testis weight with age contrasted, rather artificially, with the significantly positive correlation obtained with absolute weights. In Section 6.3 it has already been noted that absolute testis weight was found to be uncorrelated with body weight in CBA mice, and their hybrid crosses. It was probable, therefore, that division of absolute weight by body weight could be misleading, in these strains.

Although relative testis weight was found to decrease with age, significantly in both the F_1 's, O and N, the slope of the former seemed slightly more negative. In this respect, $F_1(0)$ was similar to backcrosses OD and OC in possessing a more positive testis growth trend than its reciprocal cross. The possibility of this representing an indirect maternal influence, resulting in similar expression of this sex-limited character in males of successive generations, was complicated by the fact that F_1 's bore an inverse, rather than a direct, relationship with males of their maternal strains, with respect to correlation with age. It was possible that the more negative correlations of F_1 testis weights (especially relative weights) with age, compared with either parental strain, signified a breakdown in co-adaptedness which in DBA and C57 ensured testis weight (spermatogenesis) was maintained with age, by different means.

Friedman two-way analyses of variance were performed on absolute and relative spleen dry weight means, for certain age groups, for different combinations of parental and backcross strains. The results of these tests are given in Table 6.48.

Table 6.48 Probabilities (%'s) for Friedman two-way analyses of variance between absolute and relative testis weight means of different age groups and strains.

Strains	Age periods	p(%) absolute weights	p(%) relative weights
DBA, CBA, C57	8, 12, 14, 16	0.46	0.46
DBA, O, N, C57	10, 12, 14, 16	0.62	0.16
DBA, OD, O	10, 12, 14, 16	4.20	0.46
DBA, ND, N	10, 12, 14, 16	6.90	0.46
DBA, OD, ND, O, N	10, 12, 14, 16	2.50	2.00
C57, OC, O	12, 14, 16	NS	NS
C57, NC, N	12, 14, 16	NS	2.80
C57, OO, NC, O, N	12, 14, 15	NS	NS

Although fewer age periods were sampled than for backcrosses to DBA, backcrosses to C57 seemed to have had statistically similar growth trends to C57, whereas those to DBA appeared to possess testes that had quite different growth patterns from, particularly, DBA. This evidence, as well as the fact that DBA's were found to have, unlike any other strain, testis weights that increased markedly between 8 and 16 weeks, even when

corrected for body weight, suggested that these mice were exceptional with respect to androgen production.

6.6.1.2 Testis dry weight differences between strains within age groups.

The results of Kruskal-Wallis one-way analyses of variance, performed on absolute and relative testis dry weight values, for related strains, for each age group, are given in Table 6.49. It was clear that significant differences, in both relative and absolute weights, existed between the three parental strains, and their hybrids. The significances of comparisons between N, CN and C57, and between DBA, QD and Q, differed, radically, depending on whether absolute or relative weights were used. Relative testis weight has been shown, already, in Section 6.6.1.1 to be an inappropriate statistic to use for strain CBA, and its hybrids. From the K-W result for absolute weight, it was concluded that DBA, QD and Q testes were of statistically similar weights, at 12 weeks.

Although absence of correlation between absolute testis weight and body weight (Section 6.3) indicated the potential for distortion by division of testis weight by body weight, in such strains, it did not, necessarily, follow that the presence of a significant correlation signified the necessity for body weight correction; it being impossible without further endocrine information to distinguish between anabolic effects of testosterone on body weight, and the co-ordinated general growth control exerted by the pituitary and other glands. Testosterone has been shown in DBA/2J and C57BL/10J castrated males to restore body weight to normal in the latter strain, while resulting in only a partial recovery

Table 6.49

Kruskal-Wallis one-way analysis of variance of absolute and relative testis dry weights for combinations of strains for each age group (all probabilities expressed as %'s).

Age (Weeks): Strains	Absolute Testis Weights					Relative Testis Weights				
	10	12	14	16		10	12	14	16	
DBA, CBA, C57	0.05*	0.2	0.05	0.1		0.05*	0.05	0.05	0.05	
DBA, Q, CBA	0.05*	0.1	-	-		0.05*	0.1	-	-	
DBA, N, O, C57	3.1	9.0	0.1	2.1		0.1	0.1	0.05	0.05	
DBA, OD, O	6.4	2.1	0.4	NS		NS	1.2	6.4	0.05	
DBA, ND, N	NS	1.3	0.7	2.6		NS	NS	0.2	0.6	
DBA, OD, ND, O, N	7.60	0.2	0.1	4.9		NS	7.2	1.5	0.05	
O, OC, C57	-	1.2	0.05	0.1		-	4.1	0.05	0.05	
N, NC, C57	-	NS	6.4	1.7		-	2.6	0.1	0.2	
O, N, OC, NC, C57	-	2.3	0.05	0.1		-	2.7.	0.1	0.05	
OC, NC, CN	-	-	NS	-		-	-	NS	-	
N, CN, C57	NS	-	NS	-		3.0	-	0.2	-	
DBA, QD, Q	-	7.8	-	-		-	0.7	-	-	
Q, QB, CBA	-	2.2	-	-		-	2.2	-	-	

* These comparisons were from the 8 weeks age group.

to control weight in the former (Bartke, 1974). When testis and body weight data from a study of 26 inbred strains of mouse (West et al., 1980) were analysed using non-parametric correlation, an overall negative coefficient was obtained, that was not statistically significant. It thus seemed that, although significant positive correlations might be found within strains, such as DBA/2J and C57BL/Tb in the present work, differences in other factors affecting body weight, between strains, resulted in a loss in correlation with testis weight. It was thus thought that, in the absence of evidence concerning the causality of the positive correlations, observed between testis and body weights in most strains (see Section 6.3), each weight statistic would have to be judged on its individual merit, particularly with respect to producing phenotypic segregations that were realistic, in that improbable genetic models were not required to explain them.

Table 6.50 contains the probabilities of Mann-Whitney U tests performed on pairs of strains for each age group. Absolute and relative testis dry weights of CBA were found to be highly significantly lighter than those of DBA or C57, for all age periods. Although C57 relative testis weight was also lower than that of DBA for every age, absolute weights of the two strains became significantly different only in the 16-week age group. A M-W 2-tail test between all DBA and C57 values, from all age groups, however, gave a probability of $p = 0.0156$, so it could be concluded that C57's had significantly lighter testes, even on the basis of absolute weight.

Strains N, OD and NC had similar absolute testis dry weights as DBA and reciprocal strains O, ND and OC possessed heavier absolute weights. None of the backcrosses resembled C57 when absolute weights

Table 6.50 Mann-Whitney two-tailed probabilities for tests between testis weights of pairs of strains within each age group (all probabilities expressed as %'s).

Age (Weeks): Tests	Absolute Testis Weights				Relative Testis Weights			
	10	12	14	16	10	12	14	16
DBA v. CBA	0.009*	0.009	0.009	0.07	0.009*	0.009	0.009	0.02
DBA v. C57	8.0	NS	NS	1.07	0.01	0.03	0.009	0.009
C57 v. CBA	0.009*	0.02	0.009	0.08	0.02*	0.33	0.009	0.02
DBA v. O	7.14	3.5	0.05	NS	NS	0.86	1.30	0.009
DBA v. N	NS	4.86	NS	NS	NS	NS	0.02	0.06
DBA v. Q	0.48	3.81	-	-	0.04	0.95	-	-
C57 v. N	NS	NS	NS	NS	1.60	0.61	0.009	0.22
C57 v. O	0.95	NS	0.009	2.46	3.81	NS	0.08	0.77
CBA v. Q	1.47*	9.52	-	-	1.47*	9.52	-	-
OD v. DBA	0.88*	NS	NS	NS	0.44*	3.90	NS	1.02
OD v. O	7.14	0.40	0.68	NS	NS	NS	NS	3.13
ND v. DBA	NS	0.37	0.56	6.67	NS	NS	2.05	NS
ND v. N	NS	NS	0.37	1.40	NS	NS	NS	NS
OC v. O	-	3.81	3.00	NS	-	NS	NS	NS
OC v. C57	-	0.64	0.08	0.01	-	2.0	0.04	0.009
NC v. N	-	NS	NS	4.20	-	7.57	NS	NS
NC v. C57	-	NS	2.53	0.99	-	NS	3.25	0.25
CN v. N	NS	-	NS	-	2.27	-	NS	-
CN v. C57	NS	-	8.0	-	NS	-	NS	-
OC v. NC	-	0.24	NS	NS	-	NS	NS	NS
OC v. CN	-	-	NS	-	-	-	NS	-
NC v. CN	-	-	NS	-	-	-	NS	-
N v. O	NS	NS	0.08	NS	NS	8.05	NS	8.13
OD v. ND	NS	0.09	NS	1.60	NS	NS	NS	NS
QB v. CBA	-	5.19	4.85	-	-	5.19	NS	-
QB v. Q	0.009	7.14	-	-	0.009	7.14	-	-
QD v. DBA	-	NS	8.18	-	-	1.22	0.03	-
QD v. Q	-	NS	-	-	-	NS	-	-
ND v. QD	-	0.22	0.39	-	-	2.31	6.37	-

* These comparisons were from the 8 weeks age group.

were considered. More meaningful phenotypic segregation was obtained using relative weights. $F_1(O)$ and (N) relative weights resembled those of DBA although their negative growth trends, discussed in the previous section, caused them to become considerably lighter than DBA values by 16 weeks. Both hybrids were significantly heavier than C57, in respect of relative weight. Backcrosses were intermediate with respect to F_1 values and parental strain values. Very little difference appeared to exist between the three C57 backcrosses; strains tending to be significantly different from their C57, rather than their F_1 , parent. The relative testis weights of CN, although slightly lighter than those of NC, were not significantly so; a M-W 2-tail test carried out on all data for each strain failing to demonstrate any difference.

Correction of absolute testis weights for body weight appeared to cause significant differences between reciprocal strains to disappear. This was due to marked differences in body weights of reciprocal crosses. Table 6.51 gives the body weights of DBA, C57 and their hybrid strains, for each age group.

Table 6.51 Mean body weights for different strains and age groups
(Body weight (Gms x 10)).

Age (weeks)	8	10	12	14	16
Strain					
DBA	259 (12)	251 (10)	265 (19)	264 (12)	283 (15)
C57	294 (11)	292 (20)	323 (14)	327 (16)	350 (13)
$F_1(O)$	-	323 (2)	333 (5)	352 (7)	354 (8)
$F_1(N)$	-	280 (4)	303 (17)	298 (19)	305 (6)
OD	237 (3)	252 (10)	274 (8)	291 (10)	300 (17)
ND	-	266 (8)	306 (21)	325 (13)	317 (7)
OC	-	-	334 (6)	333 (19)	346 (20)
NC	-	-	321 (15)	329 (13)	340 (10)
CN	-	303 (13)	-	364 (4)	376*(6)

* these animals 19 weeks old.

Body weight appeared to follow a similar pattern to corticosterone productive capacity (R) in that a negative maternal influence appeared to operate between parental, F_1 and DBA backcross generations. M-W 2-tail tests between all values for OD and ND gave $p = 0.00006$ whereas no significant difference was observed between OC and NC (CN was also not significantly different from NC).

It was concluded, therefore, that good evidence existed of strain differences in testis weight (absolute and relative) between the three parental strains. The sharp difference between DBA, QD, Q absolute weights and those of QB and CBA supported previous evidence (Hayward and Shire, 1974) that a major genetic effect, contributing to small testis size in strain CBA, originates from the male sex chromosome. The difference between DBA and C57 appeared to be caused by DBA-dominant factor(s) whose interaction(s) with a possible maternal influence were indistinguishable from maternal interactions affecting body weight, in general.

6.6.2 Comparisons of testis dry weight variances.

Tables 6.52 and 6.53 give the individual age group variance estimates, for each strain, as well as the overall strain estimates formed from values centred on zero (A), and from uncentred values (B). Table 6.54 gives the ratios of variance of backcrosses over those of parental values, and the probabilities of F tests carried out on these ratios. From this table, it can be seen that, for all tests, QD variances were significantly higher than those of either Q or DBA. QB, on the other hand, possessed variance values that were similar to parental types. These results suggest that in addition to the gross effect of the Y chromosome-linked factor, referred to in the last section,

Table 6.52 Absolute testis dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group mean.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	110 (12)	232 (6)	237 (19)	169 (12)	99 (14)	158 (63)	218 (63)
CBA	42 (15)	-	20 (5)	62 (8)	30 (4)	38 (32)	111 (32)
C57	619 (11)	600 (19)	814 (14)	410 (15)	128 (13)	491 (72)	558 (72)
Q	2 (2)	117 (9)	53 (2)	-	-	83 (13)	101 (13)
O	-	3 (2)	4 (4)	272 (7)	187 (8)	148 (21)	254 (21)
N	-	149 (4)	472 (17)	229 (17)	90 (6)	282 (44)	312 (44)
OD	56 (3)	225 (6)	218 (8)	681 (13)	319 (17)	349 (47)	394 (47)
ND	-	512 (8)	299 (16)	405 (12)	139 (7)	318 (43)	433 (43)
OC	-	-	161 (6)	275 (19)	348 (20)	281 (45)	317 (45)
NC	-	-	935 (15)	301 (13)	453 (10)	562 (38)	608 (38)
CN	-	255 (13)	-	898 (4)	332 (6)	337 (23)	546 (23)
QD	-	-	558 (10)	213 (5)	287 (7)	362 (22)	448 (22)
QB	-	79 (10)	23 (6)	5 (4)	-	44 (20)	64 (20)

Table 6.53 Relative testis dry weight variances for each strain and age group and total variances obtained with (A) and without (B) correction for age group mean.

Age (Weeks):	8	10	12	14	16	Total Variance A	Total Variance B
Strain							
DBA	134 (12)	386 (6)	155 (19)	159 (12)	77 (14)	144 (63)	179 (63)
CBA	129 (15)	-	25 (5)	76 (8)	51 (4)	84 (32)	302 (32)
C57	485 (11)	400 (19)	950 (14)	202 (15)	92 (13)	399 (72)	410 (72)
Q	150 (2)	57 (9)	33 (2)	-	-	53 (13)	105 (13)
O	-	31 (2)	42 (4)	133 (7)	77 (8)	75 (21)	177 (21)
N	-	162 (4)	302 (17)	328 (17)	192 (6)	246 (44)	286 (44)
OD	312 (3)	311 (6)	253 (8)	896 (13)	366 (17)	447 (47)	654 (47)
ND	-	325 (8)	411 (16)	339 (12)	142 (7)	310 (43)	319 (43)
OC	-	-	256 (6)	179 (19)	358 (20)	257 (45)	295 (45)
NC	-	-	2069 (15)	534 (13)	241 (10)	1015 (38)	1021 (38)
CN	-	159 (13)	-	857 (4)	123 (6)	232 (23)	269 (23)
QD	-	-	632 (10)	295 (5)	356 (7)	429 (22)	546 (22)
QB	-	66 (10)	55 (6)	12 (4)	-	48 (20)	133 (20)

Table 6.54 Variance ratios for absolute and relative testis dry weight using variance estimates that were corrected (A) and uncorrected (B) for age group means.

Strain	Absolute Testis Weight				Relative Testis Weight			
	Ratio		Ratio		Ratio		Ratio	
Ratio	Var. A's	p (%)	Var. B's	p (%)	Var. A's	p (%)	Var. B's	p (%)
OD/DBA	2.21	0.5	1.81	2.5	3.10	0.1	3.65	0.1
ND/DBA	2.01	0.5	1.99	1.0	2.15	0.5	1.78	2.5
QD/DBA	2.29	1.0	2.06	2.5	2.98	0.1	3.05	0.1
OD/O	2.36	5.0	1.55	NS	5.96	0.1	3.69	0.5
ND/N	1.13	NS	1.39	NS	1.26	NS	1.12	NS
QD/Q	4.36	1.0	4.44	1.0	8.09	0.1	5.20	0.5
OC/C57	0.57	NS	0.57	NS	0.64	NS	0.72	NS
NC/C57	1.14	NS	1.09	NS	2.54	0.1	2.49	0.1
CN/C57	0.69	NS	0.98	NS	0.58	NS	0.66	NS
QB/CBA	1.16	NS	0.58	NS	0.57	NS	0.44	NS
OC/O	1.90	NS	1.25	NS	3.43	0.5	1.67	NS
NC/N	1.99	2.5	1.95	2.5	4.13	0.1	3.57	0.1
CN/N	1.20	NS	1.75	NS	0.94	NS	0.94	NS
QB/Q	0.53	NS	0.63	NS	0.91	NS	1.27	NS
<u>BC (D)</u> PAR.	1.69	2.5	1.62	2.5	2.29	0.1	2.31	0.1
<u>BC (C)</u> PAR.	1.05	NS	1.08	NS	1.73	1.0	1.63	2.5
<u>All BC</u> All PAR.	1.20	NS	1.21	NS	1.81	0.5	1.82	0.5

BC (D)
PAR. = pooled (OD and ND) / pooled (DBA, O and N)

BC (C)
PAR. = pooled (OC, NC and CN) / pooled (C57, O and N)

All BC
All PAR. = pooled (OD, ND, OC, NC and CN) / pooled (DBA, O, N and C57)

DBA and CBA differ in other ways, genetically, that appear to be CBA - dominant.

When absolute weights were used, differences between DBA and C57 appeared to reveal themselves through increased variances in backcrosses to DBA, both considered individually and when pooled. No significant effects, in either individual or pooled ratios, could be found for absolute weight variances of C57 backcrosses.

Relative weights, however, showed significantly greater variances in backcrosses to both parents and the ratio of the variance estimate for all segregating lines to that of all parental strains was significant for relative, but not for absolute, weights.

It was, thus, concluded that the significantly greater degrees of variation observed in backcrosses, particularly to DBA, compared with those of parental lines, indicated the presence of genetic segregation between DBA and the other two strains, affecting testis size. Differences were more marked in DBA backcrosses, probably reflecting differences in androgen metabolism between those strains and C57 backcrosses.

6.6.3 Testis weights and growth trends associated with values selected for hetero- or homo-zygosity at the *Idh-1*, *Mod-1*, *d*, *b* and *a* genetic loci.

6.6.3.1 Marker-linked influences on testis growth.

Tables 6.55 and 6.56 give the K-W analysis of variance and correlation statistics for values selected according to *Idh-1* type, in the manner described before, for thymus and spleen weight.

Marked differences in correlation statistics were apparent for testis absolute dry weights of different allotypes. This was present,

Table 6.55 Mean absolute testis weights for different Idh-1 types and age groups
(Absolute Weights (MGMS.) x 10.)

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Id Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
OD	Hom.	156 (2)	152 (3)	132 (5)*	160 (7)	NS	+0.047	NS, NS	-5
OD	Het.	134 (4)	133 (4)	169 (5)*	149 (9)	4.4	+0.427	240, 564	+54
ND	Hom.	144 (4)	157 (3)	180 (7)	174 (6)	4.1	+0.504	120, 50	+73
ND	Het.	149 (4)	173 (13)	170 (5)	168 (1)	NS	+0.385	350, NS	+34
OC	Hom.	-	185 (2)	165 (10)	182 (9)	6.4	+0.178	NS, NS	+28
OC	Het.	-	188 (4)	173 (9)	168 (11)	6.4	-0.435	170, NS	-49
NC	Hom.	-	161 (9)	166 (5)	179 (4)	NS	+0.189	NS, NS	+58
NC	Het.	-	142 (6)	168 (6)	161 (6)	NS	+0.323	960, 733	+111
CN	Hom.	148 (4)	-	167 (2)	160 ^a (4)	NS	+0.138	NS, NS	+21
CN	Het.	141 (9)	-	189 (2)	172 ^a (2)	6.4	-0.006	NS, NS	+9

Table 6.56 Mean relative testis weights for different Idh-1 types and age groups
(Relative Weights x 10 (mgms./100gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Id Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
OD	Hom.	578 (2)	545 (3)	469 (5)	536 (7)	NS	-0.438	350, 250	-26
OD	Het.	556 (4)	493 (4)	596 (5)	501 (9)	4.6	-0.082	NS, NS	-8
ND	Hom.	549 (4)	484 (3)*	561 (7)	555 (6)	NS	+0.252	NS, NS	+8
ND	Het.	547 (4)	579 (13)*	509 (5)	502 (1)	NS	-0.177	NS, 272	-23
OC	Hom.	-	543 (2)	493 (10)	498 (9)	NS	-0.321	780, NS	-6
OC	Het.	-	571 (4)	521 (9)	510 (11)	NS	-0.373	360, 780	-16
NC	Hom.	-	522 (9)	523 (5)	516 (4)	NS	-0.032	NS, NS	-5
NC	Het.	-	441 (6)	524 (6)	476 (6)	NS	+0.181	NS, NS	+26
CN	Hom.	487 (4)	-	472 (2)	439 ^a (4)	NS	-0.471	850, NS	-5
CN	Het.	464 (9)	-	512 (2)	430 ^a (2)	NS	-0.386	960, 488	-11

* M-W 2-tail probability for difference between Id types of same age = 2.5%.

^a These animals 19 weeks old.

to similar extents, in backcrosses to both parental strains.

Consistent differences between reciprocal strains indicated that DBA-derived factor(s) were more influenced by maternal environment than those from C57. The possibility of a sex chromosome-linked effect exerting a modulating influence on the expression of the Idh-1-linked effect, was suggested by the lack of difference, in correlation and slope, exhibited between CN allotypes compared with NC, although caution was used in interpreting this, as with all observations concerning CN, on account of small sample size, and the presence of mice of non-comparable ages.

Relative weights showed reduced differences in age correlations, that were of significant proportions only in backcrosses to DBA. The lack of difference, observed for absolute CN weights, was found also with relative weights. Maternal influence appeared to interact in an identical, although reduced, manner to that observed for absolute weights. The factor(s), linked to the DBA-derived allele of Idh-1, appeared to be associated with more negative growth trends, compared with the other allotype, in offspring of $F_1(0)$ dams, while, in progeny of $F_1(N)$ dams, their action(s) seemed to be totally reversed; being attended by increased, positive growth trends.

Absolute and relative testis weight means, with correlation statistics, for values selected according to Mod-1 type are given in Tables 6.57 and 6.58. The different weight statistics can be observed to possess similar types of difference between Mod-1 types, the only important difference being that with relative weights, backcrosses to C57 appeared to show a more marked difference in correlation and slope between allotypes compared to DBA backcrosses. Differences were, in

Table 6.57 Mean absolute testis weights for different Mod-1 types and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Mod Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
OD	Hom.	152 (1)	142 (3)	157 (5)	155 (10)	NS	+0.074	NS, NS	-2
OD	Het.	139 (5)	144 (5)	144 (5)	152 (6)	NS	+0.078	NS, NS	+8
ND	Hom.	147 (6)	168 (6)	181 (3)	174 (3)	8.4	+0.580	60, 176	+65
ND	Het.	144 (2)	170 (10)	174 (9)	173 (4)	NS	+0.265	NS, NS	+32
OC	Hom.	-	191 (3)	169 (14)	179 (7)	NS	+0.087	NS, NS	+32
OC	Het.	-	184 (3)	167 (5)	171 (13)	NS	-0.403	350, 766	-40
NC	Hom.	-	148 (6)	172 (6)	171 (6)	NS	+0.236	NS, 895	+95
NC	Het.	-	156 (9)	155 (4)	163 (4)	NS	+0.128	NS, NS	+41
CN	Hom.	141 (11)	-	174 (3)	-	NS	-0.148	NS, NS	-49
CN	Het.	151 (2)	-	192 (1)	-	-	+1.00	- -	+132

Table 6.58 Mean relative testis weights for different Mod-1 types and age groups
(Relative Weights x 10 (mgms./100gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	Mod Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ⁻²)
OD	Hom.	644 (1)	530 (3)	568 (5)	518 (10)	8.6	-0.525	70, 19	-29
OD	Het.	547 (5)	520 (5)	497 (5)	512 (6)	NS	-0.294	920, 311	-22
ND	Hom.	552 (6)	556 (6)	567 (3)	558 (3)	NS	+0.195	NS, NS	+1
ND	Het.	538 (2)	565 (10)	530 (9)	539 (4)	NS	-0.086	NS, NS	-7
OC	Hom.	-	585 (3)	509 (14)	534 (7)	NS	+0.070	NS, NS	+1
OC	Het.	-	539 (3)	499 (5)	488 (13)	NS	-0.456	190, 303	-17
NC	Hom.	-	454 (6)	541 (6)	496 (6)	NS	-0.014	NS, NS	+23
NC	Het.	-	514 (9)	498 (4)	486 (4)	NS	-0.025	NS, NS	-10
CN	Hom.	467 (11)	-	472 (3)	-	NS	-0.367	980, NS	-13
CN	Het.	496 (2)	-	551 (1)	-	-	+1.00	- -	+19

general, less marked than those observed with Idh-1, and maternal interaction, if assessed by differences in slopes of weight increase, appeared negligible in backcrosses to C57 but the DBA-derived allele appeared to be linked to a larger correlation difference in DBA backcrosses.

A modifying influence by a sex-chromosome-linked character was a possible interpretation of the reversed relationships observed for backcross CN, although with sample size even smaller than those for Idh-1, this conjecture had to be even more speculative.

The effects of d type on absolute and relative testis dry weight means can be seen, from Tables 6.59 and 6.60, to have been negligible;

Table 6.59 Mean absolute testis dry weights for different d types and age groups (Absolute Weights (MGMS.) $\times 10$).

Age (Weeks):		10	12	14	16	K-W Across Ages				
Strain	d Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ⁻³)	
OD	Hom.	137 (3)	142 (3)	154 (7)	154 (12)	NS	+0.215	NS, NS	+12	
OD	Het.	145 (3)	144 (5)	146 (6)	157 (5)	NS	+0.076	NS, NS	+9	
ND	Hom.	150 (5)	170 (8)	178 (4)	169 (2)	NS	+0.472	210, 467	+50	
ND	Het.	140 (3)	170 (8)	175 (8)	175 (5)	9.0	+0.358	430, 688	+51	
QD	Hom.	-	139 (5)	132 (3)	156 ^a (3)	NS	+0.279	NS, 743	+31	
QD	Het.	-	139 (5)	153 (2)	161 ^a (4)	NS	+0.383	NS, 722	+35	

Table 6.60 Mean relative testis dry weights for different d types and age groups (Relative Weights $\times 10$ (mgms./100gms. bwt. $\times 10$)).

Age (Weeks):		10	12	14	16	K-W Across Ages				
Strain	d Type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)	
OD	Hom.	584 (3)	530 (3)	529 (7)	518 (12)	NS	-0.359	330, 28	-26	
OD	Het.	543 (3)	520 (5)	512 (6)	521 (5)	NS	-0.317	870, 476	-21	
ND	Hom.	564 (5)	567 (8)	545 (4)	550 (2)	NS	-0.100	NS, NS	-6	
ND	Het.	522 (3)	556 (8)	536 (8)	546 (5)	NS	+0.062	NS, NS	0	
QD	Hom.	-	466 (5)	450 (3)	413 ^a (3)	NS	-0.544	420, 951	-9	
QD	Het.	-	518 (5)	505 (2)	417 ^a (4)	NS	-0.430	930, 434	-14	

^a These animals 24 weeks old.

Table 6.61 Mean absolute testis dry weights for different b types and age groups
(Absolute Weights (MGMS.) x 10).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	b type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
OD	Hom.	137 (3)	134 (2)	142 (5)	152 (8)	NS	+0.037	NS, NS	-9
OD	Het.	145 (3)	146 (6)	155 (8)	158 (9)	NS	+0.331	490, 837	+44
ND	Hom.	148 (3)	178 (9)	175 (4)	190 (1)	NS	+0.455	330, NS	+54
ND	Het.	145 (5)	159 (7)	177 (8)	170 (6)	5.0	+0.441	120, 29	+64
QD	Hom.	-	138 (7)	143 (3)	168 ^a (3)	NS	+0.533	30, 178	+51
QD	Het.	-	142 (3)	136 (2)	152 ^a (4)	NS	+0.114	NS, NS	+20

Table 6.62 Mean relative testis dry weights for different b types and age groups
(Relative Weights x 10 (mgms./100gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	b type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
OD	Hom.	584 (3)	519 (2)	500 (5)	514 (8)	NS	-0.433	250, 22	-31
OD	Het.	543 (3)	525 (6)	534 (8)	523 (9)	NS	-0.173	NS, NS	-6
ND	Hom.	556 (3)	586 (9)	529 (4)	598 (1)	NS	-0.079	NS, NS	-7
ND	Het.	544 (5)	530 (7)	544 (8)	539 (6)	NS	+0.056	NS, NS	0
QD	Hom.	-	493 (7)	492 (3)	434 ^a (3)	NS	-0.223	NS, NS	-9
QD	Het.	-	489 (3)	442 (2)	401 ^a (4)	NS	-0.551	620, 464	-13

Table 6.63 Mean absolute testis dry weights for different a types and age groups
(Absolute Weights (MGMS.) x 10.)

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	a type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ³)
QD	Hom.	-	133 (5)	142 (3)	181 ^a (1)	NS	+0.754	100, 43	+78
QD	Het.	-	145 (5)	138 (2)	155 ^a (6)	NS	+0.114	NS, NS	+21

Table 6.64 Mean relative testis dry weights for different a types and age groups
(Relative Weights x 10 (mgms./100gms. bwt. x 10)).

Age (Weeks):		10	12	14	16	K-W Across Ages			
Strain	a type					p (%)	r(NPAR)	p (%x10 ²) NPAR, PAR	Slope (x10 ²)
QD	Hom.	-	467 (5)	492 (3)	469 ^a (1)	NS	+0.293	NS, NS	+1
QD	Het.	-	516 (5)	441 (2)	406 ^a (6)	NS	-0.557	240, 217	-17

^a These animals 19 weeks old.

supporting the observation, made with Mod-1 selected data, that a possible chromosome 9 effect on testis weight appeared to be more penetrant in backcrosses to C57. The possibility of a similar penetrance effect in QD's therefore, made it impossible to be sure that CBA did not differ from DBA, with respect to the Mod-1 and d-linked effect, in the same way as C57.

Mean absolute and relative testis weights selected for b type, as shown in Tables 6.61 and 6.62, showed no perceptible difference in trends for ND mice, but OD weights demonstrated more positive trends (less negative, in the case of relative weight) with age, in animals heterozygous for the C57-derived allele. Although this relatively small difference in OD correlations could have been a random occurrence, this was thought to be an unlikely explanation due to a similar size of difference being apparent between different b allotypes in QD's. Interaction(s) with other gene difference(s) and/or maternal effects were thought to account for the difference between ND and QD with respect to the b-linked effect.

It was conceivable that such a gene difference, existing between CBA, but possibly not C57, and DBA, may have been linked to the a locus in QD's as different allotypes, shown in Tables 6.63 and 6.64, gave different correlations of testis weight with age. The size of difference, both in terms of correlation probabilities and slope, suggested that this was not a random occurrence, statistically. The DBA-derived allele appeared to be associated with a more positive testis weight trend, having been exposed to the $F_1(Q)$ maternal environment.

In summary, it was concluded that distinct effects on testis growth trends were observed to be associated with different allotypes

of the Idh-1 and a loci, in DBA hybrids with C57 and CBA, respectively. Less pronounced, but probably significant, differences were observed for different Mod-1 and b types in backcrosses of DBA and C57. Of the two markers studied in both backcrosses, Idh-1-linked effects appeared to be more penetrant in backcrosses to DBA, whereas with Mod-1, differences were more prominent in C57 backcrosses. A little evidence existed that suggested the possible involvement of sex chromosome-linked effects that modified the expression of the Idh-1- and Mod-1-linked factors.

Maternal influences interacted with all marker-linked effects observed in reciprocal crosses, but particularly with those of Idh-1; tending to produce most change in the expression of DBA-derived alleles in backcrosses to DBA. The presence of such interactions caused the identities of the 'true' effects of the marker-linked genes to be indeterminate.

6.6.3.2 Association of marker gene allotypes with differences in testis weight.

Although significant differences between testis weights of different Idh-1 type were found, as indicated by asterisks in Tables 6.55 and 6.56, for isolated age groups of OD and ND, they were inconsistent differences with respect to all ages and both (absolute and relative) weight statistics. The only instance of an overall effect, linked to marker type, for all ages, was that found in backcross OC relative testis weights, which were found to be significantly heavier (M-W 2-tail $p = 0.0344$) for Idh-1 heterozygotes. This effect was absent in absolute weights of the same mice, which were of virtually identical mean value; suggesting the effect was largely of body weight origin.

The main reason for the absence of marker-linked effects on testis weight was thought to be the very dramatic maternal influence interactions with specific alleles, resulting in inversions of their expressions in reciprocal strains referred to in Section 6.6.3.1. Detection of significant differences between allotypes by increasing sample sizes, by pooling backcrosses, would, thus, be unlikely to be fruitful as opposing influences would diminish, rather than augment, individual strain effects. Some idea of marker-linked influences on overall testis size might be obtained, indirectly by consideration of differences in maternal interaction. Table 6.65 gives the M-W 2-tail probabilities for tests between reciprocal cross absolute testis weights selected for the same marker type.

Table 6.65 Mann-Whitney two-tailed probabilities for tests between reciprocal cross absolute testis weights of the same marker type.

Strains tested	Common allotype	M-W 2-tail p(%)
OD v. ND	Mod-1 Hom	NS
OD v. ND	Mod-1 Het	0.06
OC v. NC	Mod-1 Hom	NS
OC v. NC	Mod-1 Het	3.34

Mod-1 heterozygotes were highly significantly different between OD and ND, whereas homozygotes possessed similar absolute testis weights, in the two reciprocal strains. The effect appeared to be

reversed for OC v. NC comparisons, but it was possible that the much narrower difference in M-W test results (homozygotes: NS, heterozygotes: $p=0.033$) was not a significant one.

The fact, that no significant effects could be observed between reciprocal cross relative testis weights, bore out earlier observations concerning the nullifying effect of body weight correction on absolute testis weight differences, between reciprocal crosses.

It was thus concluded that, in the absence of consistent effects on overall testis weight, the differences in growth trends observed between allotypes were due, largely, to differences in response to trophic stimuli; a result to be expected on the basis that much of testis weight reflects rate of spermatogenesis; a much less static phenotype than reticular structure of thymus, for example.

This dynamic quality would tend not to be revealed by Mann-Whitney U tests of data of different allotypes, drawn from all age groups, as such comparisons would only reveal enduring differences, lasting over all age groups, reflecting predetermined, static elements of organ size control.

6.7 STRAIN DIFFERENCES IN CORTICOSTERONE PRODUCTIVE CAPACITY (R) CORRELATION WITH AGE.

Analysis of strain differences in the defined in vitro corticosterone production phenotype, R, in Chapter 5, considered gross, overall differences between strains, pooling values from all age groups. In the context of this chapter, the adrenal cortex can, itself, be considered as a target organ, being susceptible to alterations of function induced by interactions with other endocrine organs, particularly the testis and pituitary. The first of the following subsections briefly describes strain differences in age-related changes of corticosterone productive capacity. The following sub-section considers the contribution of marker-linked effects to such strain differences.

6.7.1 Strain differences in age-related changes in corticosterone productive capacity (R).

The mean R values, for each age group and strain, are given in Table 6.66. The accompanying statistics are identical in type and format to those given in analogous organ weight tables.

Of the three parental strains, C57 had the most negative correlation of R with age. CBA had a significant negative correlation, of smaller slope than C57, while DBA demonstrated negligible change in R with age. The three strains, thus, appeared to demonstrate greater negative trends in an order corresponding to their rankings with respect to overall size of R, observed in previous chapters.

The dramatic contrast, revealed in the trends of F_1 's(0) and (N), may have resulted from, either, the possible disruption of contrastingly co-adapted gene combinations present in the parental strains, or, the maternal effect described in Chapter 5.

Considering CBA hybrids first; QD, DBA and, possibly, Q appeared to be similar in showing no significant trend of R with age. Backcross QB, on the other hand, had correlation statistics that were undistinguishable from CBA; a pattern of strain differences similar to that observed (Section 6.6.1.1) for the Y-linked effect on testis weight.

Although backcrosses OD, ND, OC and NC all possessed trends lacking significant correlations with age, the C57-like negative correlation of backcross CN R values was regarded as being a potentially important departure. Although O and OD differed from N and ND in contrasting ways, suggestive of a negative effect of F_1 dams on their offspring, the maternal influence appeared to be positive with respect to the relationship between parental strain male correlations and those of F_1 males. An explanation for this was thought to be the action of X-chromosome-linked factor(s) that contributed to the more negative trends of R value with age, in those strains, such as N and CN, with unsegregated, C57-derived, X-chromosomes. The gross, overall differences in R value between F_1 's(0) and (N) appeared not to be due to such X-chromosome linkage, however, as variance estimates, centred on zero, to eliminate differences due to age-related changes, were very similar for the three C57 backcrosses, the following values being obtained: OC = 881 (45), NC = 919 (38) and CN = 924 (23). Although the overall R value of F_1 (0) could have been observed to be greater than that of F_1 (N) if sampling had been biased in favour of older mice of the former strain, Table 6.66 shows that the frequency distributions, of animals sampled, for each strain, from each age group, were not very different.

Ten weeks was the only age at which the mean N value was higher than that of 0; by 12 weeks the latter was significantly higher than

Table 6.66 Mean corticosterone productive capacities (R's) for different strains and age groups (Corticosterone Productive Capacity (R) $\times 10^2$ (pgms. corticosterone produced maximally/viable cell/hour $\times 10^2$)).

Age (Weeks):	8	10	12	14	16	K-W Across Ages	p (%)	r(NPAR)	p (% $\times 10^2$)	Slope
Strain						p (%)			NPAR, PAR	($\times 10^3$)
DBA	-	257 (1)	227 (15)	175 (10)	214 (6)	5.7	-0.145	NS, NS	NS, NS	-3
CBA	-	-	347 (5)	236 (4)	272 (4)	5.6	-0.390	940, NS	NS, NS	-13
C57	343 (2)	374 (7)	368 (13)	298 (10)	-	NS	-0.302	460, 521	NS, NS	-18
Q	-	220 (2)	220 (2)	-	-	-	0.00	NS, NS	NS, NS	0
O	-	207 (2)	351 (5)	378 (7)	438 (8)	NS	+0.517	70, 153	NS, NS	+69
N	-	268 (4)	251 (10)	295 (19)	270 (2)	NS	+0.160	NS, NS	NS, NS	+8
OD	188 (3)	201 (6)	178 (8)	165 (10)	189 (17)	NS	-0.019	NS, NS	NS, NS	0
ND	-	180 (5)	207 (17)	209 (13)	249 (7)	NS	+0.230	720, 578	NS, NS	+12
OC	-	-	279 (6)	272 (19)	333 (20)	NS	+0.192	NS, NS	NS, NS	+20
NC	-	-	343 (15)	328 (13)	288 (10)	NS	-0.124	NS, NS	NS, NS	-13
CN	-	370 (13)	-	406 (4)	266 ^a (6)	5.4	-0.353	500, 254	NS, NS	-21
QD	-	-	213 (9)	239 (5)	161 ^b (7)	NS	-0.073	NS, NS	NS, NS	-7
QB	-	197 (10)	242 (6)	182 (4)	-	NS	-0.383	480, 88	NS, NS	-10

Table 6.67 Mean corticosterone productive capacities (R's) for different *Idh-1* types and age groups (Corticosterone Productive Capacity (R) $\times 10^2$ (pgms. corticosterone produced maximally/viable cell/hour $\times 10^2$)).

Age (Weeks):		10	12	14	16	K-W Across Ages	p (%)	r(NPAR)	p (% $\times 10^2$)	Slope
Strain	Id Type					p (%)			NPAR, PAR	($\times 10^3$)
OD	Hom.	135 (2)	197 (3)	169 (5)	169 (7)	NS	-0.092	NS, NS	NS, NS	-4
OD	Het.	219 (7)	183 (4)	252 (5)	211 (9)	NS	+0.033	NS, NS	NS, NS	+3
ND	Hom.	201 (4)	208 (4)	200 (8)	256 (6)	NS	+0.109	NS, NS	NS, NS	+11
ND	Het.	188 (4)	207 (13)	223 (5)	210 (1)	NS	+0.171	NS, NS	NS, NS	+8
OC	Hom.	-	298 (2)	278 (10)	369 (9)	NS	+0.330	720, 932	NS, NS	+35
OC	Het.	-	269 (4)	265 (9)	304 (11)	NS	+0.057	NS, NS	NS, NS	+9
NC	Hom.	-	401 (9)	324 (5)	301 (4)	NS	-0.298	NS, 681	NS, NS	-33
NC	Het.	-	255 (6)	332 (6)	279 (6)	NS	+0.104	NS, NS	NS, NS	+20
CN	Hom.	387 (4)	-	357 (2)	257 ^a (4)	NS	-0.408	NS, 366	NS, NS	-31
CN	Het.	362 (9)	-	455 (2)	284 ^a (2)	NS	-0.309	NS, NS	NS, NS	-14

a These animals 19 weeks old.

b These animals 24 weeks old.

the former strain (M-W 2-tail $p = 0.02$). On the available evidence, therefore, it was concluded that although X-linked factor(s) played some role in modulating corticosterone productive capacity with age, it was thought that the major part of the difference in R value observed, for all ages, between F_1 's(0) and (N) was the result of a maternal influence, referred to in Chapter 5.

6.7.2 Marker-linked influences on change in corticosterone productive capacity (R) with age.

The influence of Idh-1 type can be seen from Table 6.67 to have been relatively slight. Although no significant correlations were observed it was felt that, compared to backcrosses to DBA, those to C57 demonstrated signs of an effect, with the C57-derived allele showing greatest difference between OC and NC. No significant effects were observed for allotypes of either the Mod-1 or the d loci.

Selection for b type, as shown in Table 6.68 although resulting in no significant correlations, did appear to result in differences in R trends with age in QD's and ND's, particularly in the former strain in which b heterozygotes gave a negative correlation of near significance.

Table 6.69 gives means and correlations for R values selected according to a type. Contrasting correlations were obtained for values of different allotype; the CBA-derived agouti allele appeared to result in a correlation of R with age, similar to that of CBA, indicating a degree of CBA-dominance in this effect.

In summary, the two higher corticosterone producing strains, CBA and C57, were found to possess R values that were more negatively correlated with age than DBA; C57 showing the most negative trend.

Table 6.68 Mean corticosterone productive capacities (R's) for different b types and age groups (Corticosterone Productive Capacity (R) $\times 10^2$ (pgms. corticosterone produced maximally/viable cell/hour $\times 10^2$)).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (% $\times 10^2$) NPAR, PAR	Slope ($\times 10^3$)
Strain	<u>b</u> Type								
OD	Hom.	243 (4)	177 (2)	182 (5)	206 (8)	NS	-0.074	NS, NS	-1
OD	Het.	184 (6)	178 (6)	201 (8)	173 (9)	NS	-0.081	NS, NS	-3
ND	Hom.	217 (3)	195 (10)	166 (5)	307 (1)	NS	-0.029	NS, NS	+2
ND	Het.	181 (5)	225 (7)	236 (8)	240 (6)	NS	+0.203	NS, NS	+10
QD	Hom.	-	196 (7)	250 (3)	153 ^a (3)	NS	+0.095	NS, NS	-4
QD	Het.	-	273 (2)	224 (2)	166 ^a (4)	NS	-0.575	680, 613	-16

Table 6.69 Mean corticosterone productive capacities (R's) for different a types and age groups (Corticosterone Productive Capacity (R) $\times 10^2$ (pgms. corticosterone produced maximally/viable cell/hour $\times 10^2$)).

Age (Weeks):		10	12	14	16	K-W Across Ages p (%)	r(NPAR)	p (% $\times 10^2$) NPAR, PAR	Slope ($\times 10^3$)
Strain	<u>a</u> Type								
QD	Hom.	-	163 (4)	221 (3)	255 ^a (1)	NS	+0.430	NS, NS	+13
QD	Het.	-	253 (5)	267 (2)	145 ^a (6)	7.0	-0.461	570, 365	-16

^a These animals 24 weeks old.

The differences between DBA and CBA, and DBA and C57, were, possibly, due to the action of Y- and X-linked effects, respectively, on the modulation of corticosterone productive capacity with age. Sex-linkage was not thought to be the major source of difference in overall R value between F_1 's(0) and (N).

Factors linked to the Idh-1 and b loci may have affected the relationship of R value with age in backcrosses to C57 and DBA, respectively.

A much more definite effect was observed for different a allotypes; animals possessing the CBA-derived allele showing a much greater drop in corticosterone productive potential with age than those with the DBA allele.

6.8 CORRELATIONS BETWEEN LYMPHORETICULAR TISSUE SIZE, TESTIS WEIGHT AND CORTICOSTERONE PRODUCTIVE CAPACITY (R).

The following two sub-sections describe associations detected between the four main variables measured; thymus, spleen and testis weights, and corticosterone productive capacity, R.

The first section gives the evidence of correlations existing in parental strains and also in hybrid strains unselected for genetic marker type.

The second section describes significant associations observed between variables for animals selected for different marker gene allotypes.

6.8.1 Correlations present in strains unselected for genetic marker type.

Table 6.70 gives the non-parametric correlation coefficient, r (NPAR), and the single-tailed probabilities (expressed as %'s $\times 10^2$) for this, and also the parametric, statistic for five variable combinations for each strain. No corrected spleen weights, obtained under different drying conditions, were used in this table. The thymus v. R statistics are not listed as no strain was found to exhibit a significant correlation between these two variables. Although little difference was observed in the results, if relative organ weights were used instead of absolute weights, the latter are referred to, here, as it was felt they would be less prone to artificial correlation induction due to division by a common factor.

From Table 6.70 it can be seen that the immune systems of the three parental strains appeared to differ in a number of ways. The thymuses of strain DBA animals, unlike those of CBA or C57, demonstrated

Table 6.70 Correlations between absolute organ weights for each strain.

Strain	Thymus v.		Spleen v.		Spleen v.		Thymus v.		Testis v.	
	r (NPAR)	p's % $\times 10^2$	r (NPAR)	p's % $\times 10^2$	r (NPAR)	p's % $\times 10^2$	r (NPAR)	p's % $\times 10^2$	r (NPAR)	p's % $\times 10^2$
DBA	-0.41	10 10	+0.07	NS NS	+0.13	NS NS	+0.27	210 60	-0.22	NS NS
CBA	-0.02	NS NS	-0.27	610 NS	+0.31	NS NS	-0.07	NS NS	+0.54	280 260
C57	-0.13	NS NS	+0.23	300 550	+0.27	NS NS	+0.06	NS NS	-0.09	NS NS
Q	-0.19	NS 870	+0.40	NS 950	-0.50	NS NS	-0.55	330 110	+0.80	NS NS
0	-0.04	NS NS	-0.75	60 330	+0.14	NS NS	+0.45	800 NS	-0.35	620 NS
N	+0.40	40 30	+0.43	40 1	+0.05	NS NS	+0.52	10 1	-0.23	960 560
OD	-0.06	NS NS	+0.12	NS NS	-0.35	430 NS	-0.25	NS NS	-0.14	NS NS
ND	-0.10	NS NS	+0.61	10 10	-0.20	NS NS	-0.19	NS NS	+0.07	NS NS
OC	-0.15	NS NS	+0.02	NS NS	+0.15	NS NS	-0.38	240 500	+0.24	550 440
CN	+0.04	NS NS	+0.22	NS NS	-0.43	200 220	-0.29	990 NS	-0.15	NS NS
NC	+0.02	NS NS	-0.10	NS NS	+0.26	690 940	+0.30	450 960	+0.12	NS NS
QD	+0.02	NS NS	+0.10	NS NS	-0.13	NS NS	+0.42	240 120	+0.03	NS NS
QB	+0.11	NS NS	+0.24	NS NS	-0.09	NS NS	-0.27	NS 960	+0.09	NS NS

(In each probabilities column, the probability of the $r(\text{NPAR})$ correlation coefficient is followed by that of its parametric analogue, $r(\text{PAR})$).

a significant negative correlation with absolute testis size, as well as a positive relationship with absolute spleen weight. Spleen size in C57's, on the other hand, appeared to be positively correlated with testis size, suggesting either the operation of a common pituitary influence or the possibility of a trophic influence of androgen on spleen function, in this strain.

The relationship between testis size and adrenal corticosterone productive capacity (R) appeared to be different for all strains; strain CBA revealing a significant positive relationship. The negative association observed for DBA's was found to be significant when relative testis weights were used.

Although non-parental lines showed similar correlations to parental strains in some instances, other associations appeared to be qualitatively different from any parental correlations presumably as the result of disruption of co-adapted gene combinations or due to maternal influences.

A striking difference was observed between the spleen v. testis correlations of the F_1 's(O) and (N) which could have resulted from maternal influence(s), although the positive correlation exhibited by CN, compared with OC and NC, backcross mice may have signified sex chromosome involvement. The similar, although more modest, difference observed for the reciprocal F_1 's in respect of testis v. thymus correlation may have had a similar cause.

6.8.2 Differences in correlation associated with differences in marker gene allotype.

The five correlations analysed in the previous section, in addition to the one not tabulated in Table 6.70 (thymus absolute dry weight v. corticosterone productive capacity (R)), were examined for

individual animals selected according to marker gene type. Although zero order correlations were formed initially, an ultimate aim of the analysis was to single out possible causes of effects, as far as possible, by examining partial correlations obtained by controlling for variables that were potential mediators of indirect effects. It was also hoped that some attempt could be made to evaluate the statistical significances of differences in degrees of correlation associated with different marker gene types. The fulfilment of these objectives was made possible only by resorting to parametric correlation techniques. Non-parametric correlation coefficients were, however, as have been noted in previous sections, generally, of similar sizes and significances as their parametric equivalents, and it was therefore concluded that any departure from the assumptions of normality of distribution, underlying the latter statistics, was likely to be relatively small and of little consequence in determining the main conclusions reached.

Although analyses were carried out with body weight used as a controlled variable and are referred to, later, in a specific case, the partial correlations referred to in this section (Tables 6.71 - 6.76), in general, were neither controlled for body weight nor were they formed using relative organ weights. The main reason for this decision was the inconsistency with which body weight was related to organ weights and adrenal steroid production (R), as noted already in Section 6.3. Correction for body weight under these circumstances was thought to be more likely to introduce, rather than abolish, a source of variation.

Significance of difference between correlation coefficients was ascertained by transformation to the Z distribution (Moroney, 1951), in the following fashion:

Table 6.71 Zero order and partial correlation coefficients for thymus absolute weight v. corticosterone productive capacity (R) for different marker types.

Strain	Marker State	Zero order r (PAR)	p (%x10 ²)	Part. Corr. 1	p (%x10 ²)	Part. Corr. 2	p (%x10 ²)
OD	Id HOM.	+0.329 (18)	915	+0.035 (4)	NS	+0.401 (13)	690
OD	Id HET.	-0.043 (22)	NS	+0.033 (8)	NS	-0.004 (17)	NS
ND	Id HOM.	+0.416 (16)	547	-	-	+0.816 (9)	10**
ND	Id HET.	+0.113 (22)	NS	+0.172 (11)	NS	+0.144 (17)	NS**
OC	Id HOM.	-0.240 (21)	NS	-0.108 (9)	NS	-0.129 (16)	NS
OC	Id HET.	-0.277 (23)	NS	-0.297 (9)	NS	-0.235 (18)	NS
NC	Id HOM.	-0.070 (18)	NS	-0.273 (12)	NS	-0.273 (12)	NS
NC	Id HET.	-0.072 (18)	NS	-0.081 (12)	NS	-0.070 (13)	NS
CN	Id HOM.	+0.408 (10)	NS	+0.479 (5)	NS	+0.479 (5)	NS
CN	Id HET.	-0.341 (12)	NS	-0.118 (7)	NS	-0.118 (7)	NS
OD	Mod HOM.	+0.196 (21)	NS	+0.428 (5)	NS	+0.298 (16)	NS
OD	Mod HET.	+0.092 (22)	NS	+0.276 (10)	NS	+0.242 (17)	NS
ND	Mod HOM.	-0.200 (16)	NS*	+0.003 (6)	NS	-0.126 (10)	NS*
ND	Mod HET.	+0.415 (22)	273*	+0.590 (8)	360	+0.615 (16)	30*
OC	Mod HOM.	-0.057 (24)	NS	+0.112 (7)	NS	+0.010 (19)	NS
OC	Mod HET.	-0.315 (20)	880	-0.182 (11)	NS	-0.180 (15)	NS
NC	Mod HOM.	+0.119 (18)	NS	-0.039 (12)	NS	-0.039 (12)	NS
NC	Mod HET.	-0.243 (17)	NS	-0.348 (11)	NS	-0.343 (12)	NS
CN	Mod HOM.	+0.062 (13)	NS	+0.072 (8)	NS	+0.072 (8)	NS
CN	Mod HET.	+0.724 (3)	NS	-	-	-	-
OD	d HOM.	+0.229 (27)	NS	+0.296 (8)	NS	+0.225 (22)	NS
OD	d HET.	+0.163 (20)	NS	+0.332 (7)	NS	+0.287 (15)	NS
ND	d HOM.	-0.263 (18)	NS**	-0.153 (8)	NS	-0.194 (12)	NS**
ND	d HET.	+0.479 (20)	160**	+0.514 (6)	960	+0.655 (14)	30**
QD	d HOM.	+0.232 (11)	NS	+0.104 (6)	NS	+0.104 (6)	NS
QD	d HET.	-0.441 (10)	NS	-0.695 (4)	630	-0.695 (4)	630
OD	b HOM.	-0.072 (21)	NS	-0.074 (8)	NS	-0.076 (16)	NS
OD	b HET.	+0.376 (26)	290	+0.318 (7)	NS	+0.364 (21)	440
ND	b HOM.	+0.347 (18)	790	+0.416 (6)	NS	+0.369 (11)	NS
ND	b HET.	+0.190 (20)	NS	+0.351 (8)	NS	+0.351 (15)	840
QD	b HOM.	-0.293 (13)	NS	-0.362 (7)	NS	-0.362 (7)	NS
QD	b HET.	+0.063 (8)	NS	-0.407 (3)	NS	-0.407 (3)	NS
QD	a HOM.	-0.213 (8)	NS	-0.112 (2)	NS	-0.112 (2)	NS
QD	a HET.	-0.237 (13)	NS	-0.536 (8)	550	-0.536 (8)	550

Part. corr. 1 = The partial correlation controlling for age, testis and uncorrected spleen weights.

Part. corr. 2 = The partial correlation controlling for age, testis and all spleen weights.

Correlations significantly different at the (*) 5%, (**) 2.5% levels.

Table 6.72 Zero order and partial correlation coefficients for spleen absolute weight v. corticosterone productive capacity (R) for different marker types.

Strain	Marker State	Zero Order r (PAR)	p (%x10 ²)	Part. Corr. 1	p (%x10 ²)	Part. Corr. 2	p (%x10 ²)
OD	Id HOM.	-0.649 (9)	294	-0.765 (4)	380	-0.660 (13)	40*
OD	Id HET.	-0.200 (13)	NS	-0.243 (8)	NS	-0.053 (17)	NS*
ND	Id HOM.	-0.523 (9)	744	-	-	+0.299 (9)	NS
ND	Id HET.	+0.043 (16)	NS	-0.129 (11)	NS	+0.008 (17)	NS
OC	Id HOM.	+0.406 (14)	751	+0.305 (9)	NS	+0.059 (16)	NS
OC	Id HET.	-0.071 (15)	NS	-0.177 (9)	NS	-0.043 (18)	NS
NC	Id HOM.	+0.320 (17)	NS	+0.396 (12)	810	+0.396 (12)	810
NC	Id HET.	+0.248 (17)	NS	+0.493 (12)	370	+0.601 (13)	90
CN	Id HOM.	-0.602 (10)	330	+0.105 (5)	NS	+0.105 (5)	NS
CN	Id HET.	-0.247 (13)	NS	-0.256 (7)	NS	-0.256 (7)	NS
OD	Mod. HOM.	+0.008 (10)	NS	+0.301 (5)	NS	-0.189 (16)	NS
OD	Mod. HET.	-0.533 (15)	204	-0.533 (10)	370	-0.374 (17)	570
ND	Mod. HOM.	-0.586 (12)	227	-0.649 (6)	410	-0.160 (10)	NS
ND	Mod. HET.	-0.093 (13)	NS	-0.405 (8)	NS	-0.032 (16)	NS
OC	Mod. HOM.	+0.024 (12)	NS	+0.202 (7)	NS	+0.095 (19)	NS
OC	Mod. HET.	+0.253 (17)	NS	+0.141 (11)	NS	+0.186 (15)	NS
NC	Mod. HOM.	+0.242 (17)	NS	+0.245 (12)	NS	+0.245 (12)	NS
NC	Mod. HET.	+0.282 (16)	NS	+0.312 (11)	NS	+0.334 (12)	NS
CN	Mod. HOM.	-0.347 (14)	NS	-0.359 (8)	NS	-0.359 (8)	NS
CN	Mod. HET.	+0.340 (3)	NS	-	-	-	-
OD	d HOM.	+0.024 (13)	NS*	+0.266 (8)	NS*	-0.080 (22)	NS
OD	d HET.	-0.711 (12)	50*	-0.715 (7)	150*	-0.452 (15)	340
ND	d HOM.	-0.255 (14)	NS	-0.286 (8)	NS	-0.158 (12)	NS
ND	d HET.	-0.105 (11)	NS	-0.484 (6)	NS	-0.002 (14)	NS
QD	d HOM.	+0.090 (11)	NS	+0.266 (6)	NS	+0.266 (6)	NS
QD	d HET.	-0.179 (10)	NS	+0.175 (4)	NS	+0.175 (4)	NS
OD	b HOM.	-0.054 (13)	NS	-0.039 (8)	NS	-0.069 (16)	NS
OD	b HET.	-0.363 (12)	NS	-0.305 (7)	NS	-0.265 (21)	NS
ND	b HOM.	-0.082 (12)	NS	-0.122 (6)	NS	+0.366 (11)	NS
ND	b HET.	+0.173 (13)	NS	-0.058 (8)	NS	-0.136 (15)	NS
QD	b HOM.	-0.133 (13)	NS	+0.019 (7)	NS	+0.019 (7)	NS
QD	b HET.	+0.050 (8)	NS	-0.184 (3)	NS	-0.184 (3)	NS
QD	a HOM.	-0.189 (8)	NS	-0.278 (2)	NS	-0.278 (2)	NS
QD	a HET.	-0.002 (13)	NS	+0.045 (8)	NS	+0.045 (8)	NS

Part. Corr. 1 = The partial correlation controlling for age, testis and thymus weights.

Part. Corr. 2 = The partial correlation controlling for age, testis and thymus but using all spleen values.

Correlations significantly different at the (*) 5% level.

Table 6.73 Zero order and partial correlation coefficients for thymus absolute weight v. testis absolute weight for different marker types.

Strain	Marker State	Zero Order r (PAR)	P (% $\times 10^2$)	Part. Corr. 1	P (% $\times 10^2$)	Part. Corr. 2	P (% $\times 10^2$)
OD	Id HOM.	-0.048 (18)	NS	+0.139 (4)	NS	-0.085 (13)	NS
OD	Id HET.	+0.033 (22)	NS	+0.305 (8)	NS	+0.310 (17)	990
ND	Id HOM.	-0.651 (14)	59*	-	-	-0.348 (9)	NS
ND	Id HET.	-0.024 (22)	NS*	-0.070 (11)	NS	-0.169 (17)	NS
OC	Id HOM.	-0.088 (21)	NS	+0.001 (9)	NS	-0.022 (16)	NS
OC	Id HET.	-0.053 (23)	NS	-0.175 (9)	NS	-0.076 (18)	NS
NC	Id HOM.	-0.260 (18)	NS*	-0.138 (12)	NS	-0.138 (12)	NS
NC	Id HET.	+0.374 (18)	630*	+0.298 (12)	NS	+0.311 (13)	NS
CN	Id HOM.	+0.010 (10)	NS	+0.689 (5)	430	+0.689 (5)	430
CN	Id HET.	+0.143 (12)	NS	+0.221 (7)	NS	+0.221 (7)	NS
OD	Mod HOM.	-0.296 (21)	967	-0.495 (5)	NS	-0.420 (16)	410**
OD	Mod HET.	+0.206 (22)	NS	+0.320 (10)	NS	+0.328 (17)	850**
ND	Mod HOM.	-0.279 (15)	NS	-0.095 (6)	NS	+0.163 (10)	NS
ND	Mod HET.	-0.194 (21)	NS	-0.257 (8)	NS	-0.298 (16)	NS
OC	Mod HOM.	-0.114 (24)	NS	-0.357 (7)	NS	-0.170 (19)	NS
OC	Mod HET.	+0.044 (20)	NS	-0.116 (11)	NS	-0.115 (15)	NS
NC	Mod HOM.	+0.242 (18)	NS	+0.145 (12)	NS	+0.145 (12)	NS
NC	Mod HET.	+0.016 (17)	NS	+0.075 (11)	NS	+0.053 (12)	NS
CN	Mod HOM.	-0.001 (13)	NS	-0.311 (8)	NS	-0.311 (8)	NS
CN	Mod HET.	+0.976 (3)	700	-	-	-	-
OD	d HOM.	-0.114 (27)	NS	-0.168 (8)	NS	-0.108 (22)	NS
OD	d HET.	+0.131 (20)	NS	+0.309 (7)	NS	+0.295 (15)	NS
ND	d HOM.	-0.151 (17)	NS	+0.057 (8)	NS	+0.182 (12)	NS
ND	d HET.	-0.222 (19)	NS	-0.008 (6)	NS	-0.232 (14)	NS
QD	d HOM.	-0.032 (11)	NS	+0.189 (6)	NS	+0.189 (6)	NS
QD	d HET.	+0.017 (11)	NS	+0.056 (4)	NS	+0.056 (4)	NS
OD	b HOM.	+0.186 (21)	NS	+0.179 (8)	NS	+0.174 (16)	NS
OD	b HET.	-0.162 (26)	NS	+0.026 (7)	NS	-0.036 (21)	NS
ND	b HOM.	-0.124 (16)	NS	+0.026 (6)	NS	-0.025 (11)	NS
ND	b HET.	-0.346 (20)	680	-0.315 (8)	NS	-0.272 (15)	NS
QD	b HOM.	+0.099 (13)	NS	+0.266 (7)	NS	+0.266 (7)	NS
QD	b HET.	-0.144 (9)	NS	+0.120 (3)	NS	+0.120 (3)	NS
QD	a HOM.	-0.238 (9)	NS	+0.147 (2)	NS	+0.147 (2)	NS
QD	a HET.	+0.146 (13)	NS	+0.313 (8)	NS	+0.313 (8)	NS

Part. Corr. 1 = The partial correlation controlling for age, R and uncorrected spleen weights.

Part. Corr. 2 = The partial correlation controlling for age, R and all spleen weights.

Correlations significantly different at the (*) 5%, (**) 2.5% levels.

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Table 6.74 Zero order and partial correlation coefficients for spleen absolute weight v. testis absolute weight for different marker types.

Strain	Marker State	Zero Order r (PAR)	P (%x10 ²)	Part. Corr. 1	P (%x10 ²)	Part. Corr. 2	P (%x10 ²)
OD	Id HOM.	+0.563 (9)	574	+0.794 (4)	300	+0.169 (13)	NS
OD	Id HET.	+0.084 (13)	NS	-0.107 (8)	NS	-0.200 (17)	NS
ND	Id HOM.	+0.701 (8)	265	-	-	+0.472 (9)	710
ND	Id HET.	+0.578 (16)	95	+0.575 (11)	200	+0.440 (17)	300
OC	Id HOM.	+0.268 (14)	NS	+0.182 (9)	NS	-0.165 (16)	NS
OC	Id HET.	-0.205 (15)	NS	-0.257 (9)	NS	-0.094 (18)	NS
NC	Id HOM.	-0.324 (17)	NS	-0.255 (12)	NS	-0.255 (12)	NS
NC	Id HET.	+0.086 (17)	NS	+0.368 (12)	980	+0.373 (13)	850
CN	Id HOM.	+0.590 (10)	360	+0.601 (5)	770	+0.601 (5)	770
CN	Id HET.	-0.140 (13)	NS	-0.129 (7)	NS	-0.129 (7)	NS
OD	Mod HOM.	+0.026 (10)	NS	-0.311 (5)	NS	-0.168 (16)	NS
OD	Mod HET.	+0.233 (15)	NS	-0.055 (10)	NS	-0.165 (17)	NS
ND	Mod HOM.	+0.561 (11)	360	+0.840 (6)	50	+0.555 (10)	310
ND	Mod HET.	+0.631 (13)	104	+0.690 (8)	140	+0.531 (16)	120
OC	Mod HOM.	-0.309 (12)	NS	-0.473 (7)	990*	-0.331 (19)	710**
OC	Mod HET.	+0.309 (17)	NS	+0.409 (11)	830*	+0.397 (15)	570**
NC	Mod HOM.	+0.021 (17)	NS	+0.013 (12)	NS	+0.013 (12)	NS
NC	Mod HET.	-0.347 (16)	930	-0.292 (11)	NS	-0.279 (12)	NS
CN	Mod HOM.	+0.132 (14)	NS	+0.338 (8)	NS	+0.338 (8)	NS
CN	Mod HET.	-0.592 (3)	NS	-	-	-	-
OD	d HOM.	+0.013 (13)	NS	-0.187 (8)	NS	-0.081 (22)	NS
OD	d HET.	+0.225 (12)	NS	-0.147 (7)	NS	-0.200 (15)	NS
ND	d HOM.	+0.423 (13)	750	+0.389 (8)	NS	+0.505 (12)	330
ND	d HET.	+0.793 (11)	20	+0.868 (6)	30	+0.543 (14)	150
QD	d HOM.	-0.702 (11)	80***	-0.711 (6)	240*	-0.711 (6)	240*
QD	d HET.	+0.380 (11)	NS***	+0.484 (4)	NS*	+0.484 (4)	NS*
OD	b HOM.	-0.122 (13)	NS	-0.131 (8)	NS	-0.218 (16)	NS
OD	b HET.	+0.335 (12)	NS	+0.357 (7)	NS	+0.114 (21)	NS
ND	b HOM.	+0.475 (11)	700	+0.427 (6)	NS	+0.466 (11)	540
ND	b HET.	+0.604 (13)	140	+0.513 (8)	650	+0.461 (15)	310
QD	b HOM.	+0.140 (13)	NS	+0.230 (7)	NS	+0.230 (7)	NS
QD	b HET.	-0.285 (9)	NS	-0.153 (3)	NS	-0.153 (3)	NS
QD	a HOM.	+0.056 (9)	NS	+0.357 (2)	NS	+0.357 (2)	NS
QD	a HET.	-0.083 (13)	NS	-0.151 (8)	NS	-0.151 (8)	NS

Part. Corr. 1 = The partial correlation controlling for age, R and thymus weight.

Part. Corr. 2 = The partial correlation controlling for age, R and thymus weight but using all spleen values.

Correlations significantly different at the (*) 5%, (**) 2.5% and (***) 1% levels.

Table 6.75 Zero order and partial correlation coefficients for testis absolute weight v. corticosterone productive capacity (R) for different marker types.

Strain	Marker State	Zero Order r (PAR)	P (%x10 ²)	Part. Corr. 1	P (%x10 ²)	Part. Corr. 2	P (%x10 ²)
OD	Id HOM.	-0.164 (18)	NS	+0.548 (4)	NS	+0.001 (13)	NS
OD	Id HET.	+0.015 (22)	NS	-0.028 (8)	NS	-0.013 (17)	NS
ND	Id HOM.	+0.087 (20)	NS	-	-	+0.254 (9)	NS
ND	Id HET.	+0.147 (23)	NS	+0.174 (11)	NS	+0.107 (17)	NS
OC	Id HOM.	+0.242 (21)	NS	+0.142 (9)	NS	+0.217 (16)	NS
OC	Id HET.	+0.272 (24)	994	+0.229 (9)	NS	+0.283 (18)	NS
NC	Id HOM.	-0.180 (18)	NS	-0.013 (12)	NS	-0.013 (12)	NS
NC	Id HET.	-0.074 (18)	NS	-0.294 (12)	NS	-0.327 (13)	NS
CN	Id HOM.	-0.686 (10)	140**	-0.692 (5)	420	-0.692 (5)	420
CN	Id HET.	+0.211 (13)	NS**	+0.240 (7)	NS	+0.240 (7)	NS
OD	Mod HOM.	+0.174 (21)	NS*	+0.349 (5)	NS	+0.241 (16)	NS**
OD	Mod HET.	-0.411 (22)	287*	-0.408 (10)	940	-0.472 (17)	210**
ND	Mod HOM.	-0.061 (18)	NS	+0.452 (6)	NS	-0.098 (10)	NS
ND	Mod HET.	+0.223 (25)	NS	+0.491 (8)	750	+0.288 (16)	NS
OC	Mod HOM.	+0.400 (24)	264	+0.438 (7)	NS	+0.404 (19)	350
OC	Mod HET.	+0.048 (21)	NS	+0.062 (11)	NS	+0.045 (15)	NS
NC	Mod HOM.	+0.309 (18)	NS	+0.281 (12)	NS	+0.281 (12)	NS
NC	Mod HET.	-0.287 (17)	NS	-0.154 (11)	NS	-0.151 (12)	NS
CN	Mod HOM.	+0.217 (14)	NS	+0.231 (8)	NS	+0.231 (8)	NS
CN	Mod HET.	+0.558 (3)	NS	-	-	-	-
OD	d HOM.	+0.122 (27)	NS*	+0.195 (8)	NS	+0.146 (22)	NS*
OD	d HET.	-0.389 (20)	450*	-0.411 (7)	NS	-0.477 (15)	260*
ND	d HOM.	+0.009 (19)	NS	+0.039 (8)	NS	+0.010 (12)	NS
ND	d HET.	+0.161 (24)	NS	+0.516 (6)	950	+0.187 (14)	NS
QD	d HOM.	+0.156 (11)	NS	+0.391 (6)	NS	+0.391 (6)	NS
QD	d HET.	-0.234 (9)	NS	+0.060 (4)	NS	+0.060 (4)	NS
OD	b HOM.	-0.141 (21)	NS	-0.133 (8)	NS	-0.140 (16)	NS
OD	b HET.	+0.019 (26)	NS	+0.155 (7)	NS	+0.080 (21)	NS
ND	b HOM.	+0.011 (17)	NS	+0.051 (6)	NS	-0.172 (11)	NS
ND	b HET.	+0.272 (26)	900	+0.250 (8)	NS	+0.289 (15)	NS
QD	b HOM.	-0.083 (12)	NS	+0.171 (7)	NS	+0.171 (7)	NS
QD	b HET.	-0.072 (8)	NS	+0.173 (3)	NS	+0.173 (3)	NS
QD	a HOM.	+0.547 (7)	NS	+0.433 (2)	NS	+0.433 (2)	NS
QD	a HET.	-0.290 (13)	NS	+0.037 (8)	NS	+0.037 (8)	NS

Part. Corr. 1 = The partial correlation controlling for age, thymus and uncorrected spleen weights.

Part. Corr. 2 = The partial correlation controlling for age, thymus and all spleen weights.

Correlations significantly different at the (*) 5%, (**) 2.5% levels.

Table 6.76 Zero order and partial correlation coefficients for thymus absolute weight v. spleen absolute weight for different marker types.

Strain	Marker State	Zero Order r (PAR)	p (%x10 ²)	Part. Corr. 1	p (%x10 ²)	Part. Corr. 2	p (%x10 ²)
OD	Id HOM.	-0.540 (9)	669	-0.205 (4)	NS	+0.274 (13)	NS
OD	Id HET.	-0.091 (13)	NS	+0.179 (8)	NS	+0.130 (17)	NS
ND	Id HOM.	-0.497 (9)	868	-	-	-0.356 (8)	NS*
ND	Id HET.	-0.015 (16)	NS	+0.123 (11)	NS	+0.383 (17)	530*
OC	Id HOM.	-0.208 (14)	NS	-0.062 (9)	NS	-0.066 (16)	NS
OC	Id HET.	-0.503 (14)	334	-0.569 (9)	340	-0.467 (18)	190
NC	Id HOM.	+0.322 (17)	NS	+0.346 (12)	NS	+0.346 (12)	NS
NC	Id HET.	+0.132 (17)	NS	+0.111 (12)	NS	+0.076 (13)	NS
CN	Id HOM.	-0.585 (10)	380*	-0.689 (5)	430*	-0.689 (5)	430*
CN	Id HET.	+0.240 (12)	NS*	+0.446 (7)	NS*	+0.446 (7)	NS*
OD	Mod HOM.	-0.742 (10)	70*	-0.728 (5)	320*	-0.081 (16)	NS
OD	Mod HET.	-0.029 (15)	NS*	+0.179 (10)	NS*	+0.113 (17)	NS
ND	Mod HOM.	+0.179 (12)	NS	+0.095 (6)	NS	-0.334 (10)	NS
ND	Mod HET.	-0.408 (13)	833	+0.071 (8)	NS	+0.103 (16)	NS
OC	Mod HOM.	-0.528 (12)	389	-0.603 (7)	430	-0.229 (19)	NS
OC	Mod HET.	-0.193 (16)	NS	+0.014 (11)	NS	+0.009 (15)	NS
NC	Mod HOM.	+0.279 (17)	NS	+0.363 (12)	NS	+0.363 (12)	NS
NC	Mod HET.	+0.305 (16)	NS	+0.403 (11)	860	+0.351 (12)	NS
CN	Mod HOM.	+0.265 (13)	NS	+0.447 (8)	980	+0.447 (8)	980
CN	Mod HET.	-0.403 (3)	NS	-	-	-	-
OD	d HOM.	-0.486 (13)	460	-0.354 (8)	NS	+0.033 (22)	NS
OD	d HET.	-0.109 (12)	NS	+0.181 (7)	NS	+0.054 (15)	NS
ND	d HOM.	-0.017 (14)	NS	+0.006 (8)	NS	-0.254 (12)	NS
ND	d HET.	-0.526 (11)	480	-0.118 (6)	NS	+0.084 (14)	NS
QD	d HOM.	+0.231 (11)	NS	+0.261 (6)	NS	+0.261 (6)	NS
QD	d HET.	+0.605 (12)	190	+0.464 (4)	NS	+0.464 (4)	NS
OD	b HOM.	-0.211 (13)	NS	+0.194 (8)	NS	+0.096 (16)	NS
OD	b HET.	-0.316 (12)	NS	-0.190 (7)	NS	-0.086 (21)	NS
ND	b HOM.	-0.397 (12)	NS	-0.041 (6)	NS	+0.070 (11)	NS
ND	b HET.	-0.125 (13)	NS	+0.155 (8)	NS	+0.056 (15)	NS
QD	b HOM.	+0.666 (14)	50	+0.505 (7)	830	+0.505 (7)	830
QD	b HET.	+0.190 (9)	NS	-0.063 (3)	NS	-0.063 (3)	NS
QD	a HOM.	+0.079 (10)	NS	-0.030 (2)	NS	-0.030 (2)	NS
QD	a HET.	+0.633 (13)	100	+0.492 (8)	740	+0.492 (8)	740

Part. Corr. 1 = The partial correlation controlling for age, R and testis weight.

Part. Corr. 2 = The partial correlation controlling for age, R and testis weight but using all spleen weights.

Correlations significantly different at the (*) 5% level.

$$Z = 1.15 \times \log_{10} \left(\frac{1+r}{1-r} \right)$$

Division of the difference between two z-transformed correlation coefficients by the following estimate of standard error of difference allowed a t test to be performed:

Standard error of difference between

$$\text{Z-transformed } r\text{'s} = \left(\frac{1}{N_1 - 3} + \frac{1}{N_2 - 3} \right)^{0.5}$$

where N_1 and N_2 were the degrees of freedom used in the formation of the correlation coefficients. This was the same as the sample sizes in zero order correlations but was reduced by one for every variable controlled, in the case of partial correlations. The critical t value was sought for $(N_1 + N_2 - 2)$ degrees of freedom.

Tables 6.71 to 6.76 give three series of correlation coefficients for each strain, marker gene state and organ weight correlation. In all cases, statistical differences between coefficients for different marker allotypes, within the same strain, are indicated by single, double and triple asterisks for single-tailed significances at the 5, 2.5 and 1% levels, respectively.

The first column of correlation coefficients given in each table comprises the zero order correlation coefficients for the two major variables (given in the title of each table). The column headed 'Part. Corr. 1' lists the partial correlation coefficients for the two main variables controlling for three out of the following variables (the remaining two being the main variables); age (to the nearest day) corticosterone productive capacity (R), absolute thymus dry weight, absolute spleen dry weight and absolute testis dry weight. 'Part. Corr. 1' coefficients were formed using uncorrected spleen weights, only.

The partial correlations listed under the heading 'Part. Corr. 2' differed from the first list, just described, by being computed from all spleen weight data, including those values that had been dried under different drying conditions, and corrected by the regression method described in Section 6.2.4. The computation of these latter coefficients enabled significant differences, between partial correlations of different allotypes, to be observed due to increased sample sizes.

When spleen weight was, itself, a main variable (Tables 6.72, 6.74 and 6.76) the zero order and 'Part. Corr. 1' coefficients were formed using uncorrected spleen values, while all values were used for the 'Part. Corr. 2' computations.

The total number of pairs of values used are given in parentheses after zero order coefficients, whereas the nett number of degrees of freedom are given in the cases of the two partial correlations.

The following sub-sections, entitled with the main variable correlations, followed by the list of controlled variables in parentheses, briefly describe the main results contained within Tables 6.71 - 6.76.

6.8.2.1 Thymus absolute weight v. corticosterone productive capacity, R (controlled: age, spleen and testis absolute weights).

Backcrosses to DBA in Table 6.71 showed the most significant differences in the relationship of thymus weight with R, between marker allotypes. The DBA-derived Idh-1 allele appeared to be linked to greater positive correlation of the two main variables; significantly so in backcross ND. Although CN homozygotes for this marker possessed quite different correlations from the negative values of OC and NC Idh-1 homozygotes, no significant effects were observed for this strain.

The DBA-derived chromosome 9 was indicated, by the d- and Mod-1- selected data, to be associated with greater negative correlation between thymus weight and R, especially in backcross ND.

The backcross QD d allotypes possessed different correlations, as did b allotypes of backcross OD, but in neither case was this significant. It was found, however, that when partial correlations were computed for OD b allotypes, that controlled for body weight, in addition to the other three controlled variables, coefficients were obtained that were just significantly different at the 5% level, using a single tail test (these figures are not included in Table 6.72).

6.8.2.2 Spleen absolute weight v. corticosterone productive capacity, R (controlled: age, thymus and testis absolute weights).

The DBA-derived Idh-1 allele appeared in general, from the zero order coefficients given in Table 6.72, to produce relationships, between spleen weight and R, that were of a more negative nature, particularly in backcross OD. The effect of chromosome 9 factor(s) linked to the d and Mod-1 loci was the converse of that observed for Idh-1 in the same backcross. Although QD d allotypes demonstrated no significant difference between their correlation coefficients, a difference between DBA and CBA with respect to the chromosome 9 factor could not be ruled out as the penetrance of the effect was also reduced in the analogous backcross, ND. There were no significant sex chromosome effects.

6.8.2.3 Thymus absolute weight v. testis absolute weight (controlled: age, corticosterone productive capacity, R, and spleen absolute weight).

Although significant differences in zero order correlations were obtained for Idh-1 allotypes, as can be seen from Table 6.73,

their disappearance between partial correlations suggested they were the result of indirect effect(s) exerted by one of the subsidiary variables such as thymus absolute weight.

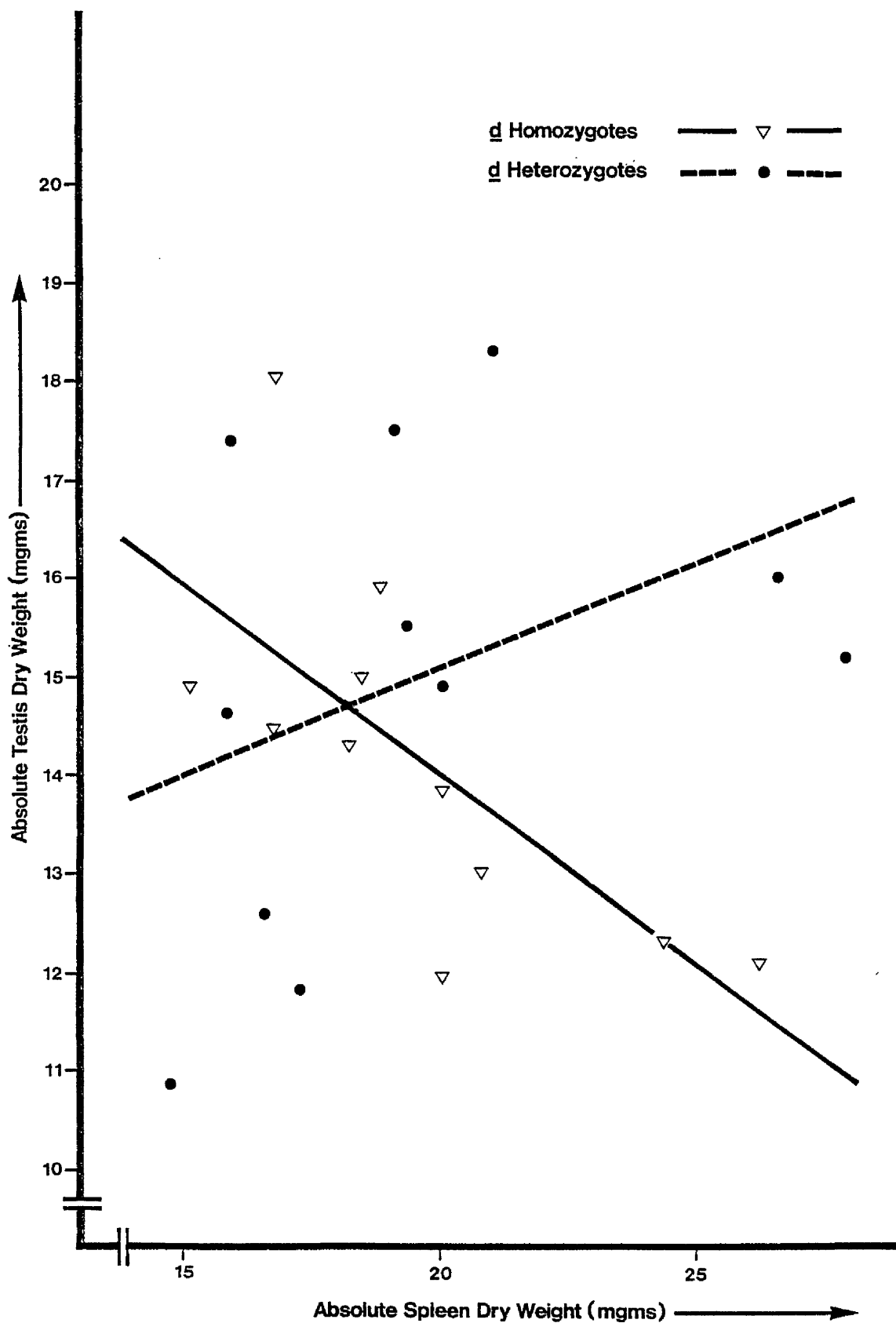
Mod-1 allotypes, on the other hand, demonstrated significantly different correlations for backcross OD animals, only after such subsidiary variables had been controlled.

Although the influence of X- or Y-linked factors appeared to have little influence on correlations of different Idh-1 types, the limited data available for Mod-1 allotypes suggested a possible sex chromosome-linked effect manifest in allotypes of this marker, in backcross CN mice.

6.8.2.4 Spleen absolute weight v. testis absolute weight (controlled: age, corticosterone productive capacity, R, and thymus absolute weight).

In contrast to the three correlations considered so far, the penetrance of a significant marker-linked effect, on the correlations of spleen with testis weight, given in Table 6.74, was greatest in backcrosses to C57; OC Mod-1 allotypes having significantly different partial correlations. Further evidence of a chromosome 9 effect on the relationship between these two variables was obtained from coefficients computed for backcross QD mice of different d type. The values forming the highly significantly different zero order correlations for these allotypes are plotted, with regression lines, in Figure 6.1. Although, strictly, the regression line for heterozygotes was inadmissible as the correlation coefficient was not significant, its inclusion in Figure 6.1 is intended to indicate the very different trends associated with differences at the d locus. Strain DBA would thus

Figure 6.1 The regression lines of absolute testis dry weight on absolute spleen dry weight for backcross QD mice of different d allotypes.



appear to differ from CBA and C57 with respect to factor(s) located on chromosome 9 controlling the relationship of spleen with testis weight; providing further evidence of the former strain's exceptional androgen requirements.

6.8.2.5 Testis absolute weight v. corticosterone productive capacity, R (controlled: age, thymus and spleen absolute weights).

From Table 6.75 it can be seen that apart from a possible sex chromosome-linked interaction present in CN mice, the Idh-1 locus did not appear to exert any noticeable effect on the relationship between R and testis weight.

All strains, with the exceptions of NC and, possibly, CN, showed evidence of d or Mod-1 allotypes possessing different testis weight v. R correlations; backcross OD significantly so.

These results suggested that the undoubted dependence of the ald phenotype on androgen for expression was probably of a qualitative ('off or on') nature rather than being of one directly dependent on quantitative aspects of adrenal sensitivity to androgen. This statement assumes that the Idh-1-linked effect observed on corticosterone productive capacity in Chapter 5 was the result of the action of an ald locus, a conclusion reached solely on the observed correlations between visible gross lipid content, Idh-1 type and R.

This topic, as well as the question of whether the Mod-1-linked effect on testis weight correlation with R reflects an alteration in sensitivity to androgen, rather than corticosterone, will be discussed more fully, shortly.

A sex-chromosome-linked effect was evident in the comparison

of the zero order correlations of CN mice selected for different Idh-1 allotypes. The partial correlations still maintained this difference, although significance was reduced, probably due to small sample size.

A similarly reduced sample size made it difficult to be certain that a factor linked to the a locus was not responsible for producing a markedly increased positive zero order correlation of testis weight with R in QD heterozygotes.

6.8.2.6 Thymus absolute weight v. spleen absolute weight (controlled: age, R and absolute testis weight).

Although common trophic influences, such as the pituitary or bone marrow, were not controlled for in the partial correlations of thymus with spleen weights, given in Table 6.76, a significant proportion of any relationship between the two organs would be likely to be the result of direct interactions involving mutual lymphocyte migration, or even trophic effects by humoral factors.

Idh-1 type appeared to influence the thymus/spleen weight relationship, especially in backcross ND, and CN values indicated the strong possibility of sex chromosome modulation of the effect; correlations for Idh-1 allotypes of this strain not only being significantly different, but also being of reversed relative sign to comparable OC and NC coefficients.

A significant Mod-1-linked effect was observed for backcross OD correlations, although the absence of difference between partial correlations using corrected as well as normal spleen data (Part.Corr.2) may have indicated, as did the d locus results, that a chromosome 9 effect, if it existed at all, was relatively small compared with that found for Idh-1.

Although the other loci, b and, particularly a, demonstrated quite large differences between many of their allotype-specific coefficients, none were of significant proportions.

6.9 CONCLUSIONS

The most important strain differences in organ weight described in this chapter were those found for the thymus. The appearance of more marked strain effects in this organ was probably due, in part, to its dramatic involution rate. The overall thymus size of strain DBA was found to be smaller than that of C57, even correcting for body weight, which was a crucial finding. It implied that the latter strain was genetically co-adapted in such a way as to ensure that the thymus was of sufficient size and resilience to be able to continue functioning normally, in the face of, presumably, high plasma stress responses of corticosterone, until animals reached old age. The variances of backcross thymus weights indicated the presence of genetic segregation that was still significant after variance as a result of involution had been minimised by using estimates centred on zero for F tests.

Mann-Whitney two-tailed U tests, carried out between pooled values for different marker gene allotypes, then indicated that two major genes appeared to influence overall thymus size, which had maximal effects in backcrosses to different parental strains. Thus, the association between, the C57-derived allele of the Mod-1 locus, and lighter thymus weight was observed, to significant extent, exclusively in backcrosses NC and CN, as well as the pooled values of (NC + CN) and (OC + NC + CN). The expression of the factor linked to the Idh-1 locus, although largely confined to DBA backcrosses, was also evident in backcross OC; an important difference between this and the Mod-1-linked gene, which will be returned to later. The DBA-derived Idh-1 allele was observed to be significantly associated with

lower thymus weight in ND, pooled (OD + ND) and OC individuals. These marker-linked factors thus had contrasting effects within the same individual, although, as has been described, the expression of one predominated over the other, depending on the backcross. If these effects are hypothesised to be the result of interaction of thymus cells with either of the two major types of steroid abounding in male mice, corticosterone and testosterone, two alternative explanations of the observations present themselves; depending on dominance and penetrance factors. It was possible, on one hand, that the Idh-1-linked effect resulted from an alteration in thymus sensitivity to testosterone which might express itself most clearly in the backcrosses with highest probable androgen levels; those to DBA. Alternatively, the chromosome 1 effect could have been the result of altered responsiveness to corticosterone which revealed itself prominently in DBA backcrosses only because its recessive nature prevented it from being expressed in the backcrosses with the highest probable corticosterone levels; those to C57. Although the situation will be seen to be less simple than these alternatives imply, a major aim of this concluding section will be to provide evidence suggesting which of these models was the most likely. Exactly the same alternatives, although in a complimentary sense, were thought to exist for the Mod-1-linked effect. Before returning to these questions, the observations concerning genetic differences in the weights of various organs studied will be reviewed, first.

The thymuses of CBA were of similar weight to those of DBA, but although the involution rates of DBA and C57 were identical, CBA was distinct from these strains in having an insignificant involution

rate, except when corrected (probably artificially) for body weight. The dimorphism between DBA and CBA thymuses thus only became distinct in later age periods, and the lack of significant difference in the variances of CBA and QB suggested that genetic differences between the two strains, in relation to thymus weight, were probably fewer than those existing between DBA and C57.

Concerning thymus involution, it was observed that DBA, $F_1(N)$, OD and OC weight losses were greatest between 14 and 16 weeks, in contrast to C57 and the other reciprocal backcrosses, which maintained relatively constant weights during this period; an effect probably resulting from maternal influence. The DBA type of late involution could have been caused by the Idh-1-linked factor as OC's displayed significant difference in the 16-week thymus weights of different allotype. The effects of the Mod-1-linked gene resulted in differences in thymus involution that were more apparent in backcrosses to C57. This fact, and the lack of any significant differences between 16-week thymus weights of homo- and hetero-zygotes for this locus, suggested that the involution mode, peculiar to DBA, was not dictated by a chromosome 9 gene. This latter site may have accounted for the different thymus involutions of CBA hybrids which revealed correlation differences for d, but not for a or b, allotypes. The latter locus appeared to be associated with greater involution rate when homozygous for the DBA allele in ND's but differences between 14 week weights were not as great as those for weights selected according to Idh-1 allotype, suggesting that the 14-16 week weight loss exhibited in DBA's was related to the expression of a factor on chromosome 1.

Turning to spleen weight, little difference was observed between the overall organ weights of strains DBA and C57, although CBA spleens were considerably lighter than the former strains. The reduced size of spleens in CBA's could not be accounted for by genes capable of segregating and engendering increased backcross variances. The facts were thought to be best fitted if the effect was regarded as originating from a Y-chromosome-linked factor. Only an outside chance of genetic segregation, directly affecting spleen size, in crosses between DBA and C57, was indicated by only two (three, if corrected ND spleens were included) significantly increased backcross variances. This was borne out by the absence of any overall marker gene effect on spleen size, with the exception of the b locus, in backcross ND, which resulted in heavier weights when homozygous for the DBA allele.

The parental strains differed in the age correlations of both absolute and relative spleen weights; following the order: CBA, DBA then C57, in increasing negativity of correlation. F_1 's(0) and (N) appeared to resemble C57 and, in general, correlations of hybrids between DBA and C57 seemed to become more negative in the rank order of their mean corticosterone productive capacities, described in the last chapter; OD's being most positively correlated with age, NC's, most negatively. The DBA-derived allele of the Idh-1 locus was associated with greater splenic weight loss; being most penetrant in backcrosses to C57. In contrast, the C57 allele of the Mod-1 marker gene was more penetrant in DBA backcrosses; resulting in greater weight decrement although this was deduced from the C57 backcrosses as maternal influences appeared to reverse the effect in OD's and ND's. In the absence of QB marker evidence, it was not possible to

be certain that CBA did not differ from DBA with respect to this chromosome 9 effect on the sole basis of there being no difference between d allotype correlations, in backcross QD. The heavier spleen weight associated with the DBA-derived b allele, referred to earlier, would appear, from age correlation differences, to be due to a greater spleen weight accretion in OD and ND individuals, homozygous for this allele. Although QD's were unlike the other two DBA backcrosses in respect of the b-linked effect, they did indicate that the DBA-derived allele of the a locus might be responsible for reduced splenic weight loss with age.

From the limited CN data, sex chromosome effects could have modulated the expression of the Mod-1-linked effect on spleen weight change, but appeared to have no influence on the Idh-1-linked effect.

Testis weight differences between DBA and C57 were similar to those reported for the same DBA line and C57BL/6J (Shire and Bartke, 1972; West et al., 1980); the latter strain having slightly smaller testes than DBA. The very much smaller organs of CBA mice have been noted before and proposed to be the result of Y-chromosome linkage (Hayward and Shire, 1974). Although absolute testis weight variances of CN fell between those of OC and NC, the relative weight variances were slightly lower than the OC/NC range. Although F_1 's (O) and (N) showed a marked body weight difference, the body weights of CN mice were not significantly different from those of OC or NC. It may be important, also, that the only age group that enabled direct variance comparisons to be made between the C57 backcrosses, showed 14-week CN variances to be as high as those of the other strains. It was thus concluded that the size and age distribution of the CN

values disallowed the possibility of sex-linkage, with respect to testis weight, from being either accepted or rejected, but it was speculated that if such linkage were present it acted in a modulatory fashion that was quite different from the dramatic effect of the Y-linked factor on CBA testis size.

The difference between DBA and C57 was apparently due to dominance of allele(s) derived from the former strain although significantly increased variances, compared with parental values, were most prominent in backcrosses to DBA, possibly due to differences between them and backcrosses to C57, with respect to plasma androgen levels. Differences between reciprocal crosses seemed to follow the same pattern as those observed for R and body weight; the differences in absolute weight often disappearing upon correction for body weight.

As no sex-linked difference was observed, in the last chapter, with respect to corticosterone productive capacity, R, this may constitute additional evidence that the absolute testis weight difference between F_1 's(0) and (N) was due to maternal influence more than X-linkage.

No evidence of marker gene-linked effects on overall testis weight was obtained; maternal interactions, small growth changes and the dynamic nature of spermatogenesis possibly accounting for this.

Examination of testis weight correlations with age revealed that, whereas DBA and C57 maintained testis weight with increasing body weight, CBA testes underwent significant reduction in size. The organs of all three F_1 hybrids lost weight in similar fashions to

CBA, possibly representing the breakdown of co-adapted processes ensuring adequate spermatogenesis, in the former hybrids, and an innate defect, though one without apparent reproductive disadvantages, in the latter parental strain (Krzanowska, 1969).

The Idh-1 and Mod-1 loci both appeared to be involved in differences in testis weight age correlation, although the effects of specific alleles were incomprehensible in the former case on account of the reversing effect of presumed maternal effects on expressions in reciprocal backcrosses to both parents. Allotype-specific differences in the case of the Mod-1 gene, however, indicated that a factor, promoting positive testis weight correlation with age, linked to the C57 allele, was most penetrant in backcrosses to C57. Reversed differences for CN allotypes, at this locus, hinted at the possibility of sex chromosome involvement.

No differences in age correlations were observed for testis weights of different d allotype in any backcross to DBA (including QD); confirming the lack of penetrance of the chromosome 9 effect in these strains.

The DBA-derived b allele appeared to result in decreased absolute testis growth with age, particularly in backcross OD. Although QD's exhibited a difference that was the reverse of that observed in the other DBA backcrosses, it was of marginal significance, whereas the DBA-derived a allele was associated with a greater positive correlation of absolute testis weight with age than that of the CBA-derived allele.

Reduction in corticosterone productive capacity, R, with age was greater in strains CBA and, particularly, C57 than in DBA. The

operation of a possible X-linked factor was thought to be a contributory cause of the difference in age correlations of the F_1 's(0) and (N), although it was possible that the greatly increased positive correlations of F_1 (0) R values could have been the result of greater trophic stimulation by the pituitary, induced as a result of maternal influence.

Although the Idh-1 and b loci were observed to possess some allotype-specific effects on the relationship of R with age, these were of a trivial nature compared with the difference observed in R correlations for different allotypes of the a gene, in QD's; the CBA-derived allele being associated with lowered corticosterone productive potential with age.

Examination of the relationships of the main variables with each other, rather than age, by formation of zero order, and partial, correlation coefficients indicated the possible cause of the a locus related effects. The most prominent differences in correlations of different agouti allotypes seem to occur in the spleen v. thymus relationship with moderate effects being observable in the thymus v. R, spleen v. testis and testis v. R correlations, as well, although no differences were found significant by t test on Z-transformed r values. It was speculated that this evidence indicated the agouti allele may be linked to a factor co-ordinating lymphoreticular system differential response to androgens and corticosteroids, possibly underlying the unusual response of CBA lymphocytes to dexamethasone (Taylor, 1976). Agouti alleles have been found to influence corticosterone plasma levels in mice (Wolff and Flack, 1971) and domesticability in rats (Milyutin, 1979).

Although the b locus appeared to exert some effect on the relationship between the thymus and R, in OD's, and between the thymus

and spleen, in QD's, these effects, as with those of the a gene were not so extreme as to give a significant t value for allotype differences.

Important differences were, however, noted for correlation coefficients of allotypes of the Idh-1 and Mod-1 genes. It was hoped that variables found to be common to pairs of correlations that had been found significant with respect to a particular marker would suggest the probable cause of linked effects. This ambition was partially fulfilled, although it was observed for many pairs of correlations that both marker-linked factors appeared to exert equally significant, although opposing, effects.

Some clues concerning differential functions of the factors was provided, however, by the findings of significant differences between partial correlations of different Idh-1 allotypes in relationships between R and the two lymphoreticular organs. Although these were accompanied by effects of equal significance for the chromosome 9 factor in the case of the thymus, the significance of difference between d allotypes, in the case of the spleen, was not maintained when all spleen values were used for the partial correlations. Such 'functional overlap' was less in evidence in correlations with testis size, and, in fact, only the Mod-1 locus (or the d locus, in the case of QD correlations with spleen weight) appeared to give significant differences between allotype partial correlations. It was thought that the dual effects observed, particularly, in the thymus, could reflect a phenomenon, already reported in the case of androgen-like action of some glucocorticoids on the submandibular glands of mice (Sato et al., 1981), which could be of more than mere biochemical

interest. One speculation might be that it could be of evolutionary advantage to allow such sharing of function between adrenal and testicular steroids, as aⁿ aversion of the threat to fitness posed by overdependence on a single class of steroids could permit the evolution of polarised physiologies in which one type of steroid predominated over the other.

Hitherto, the extensive differences between reciprocal strain correlations have not been referred to in order to allow 'genetic' effects to be examined more clearly. Maternal effects were thus thought to be best observed in situations devoid of genetic variation. In the conclusion to the last chapter, groups of DBA and C57 animals were referred to, that were near relatives in successive generations, which appeared to alternate with respect to corticosterone productive capacity. As insufficient data was obtained to form conclusions concerning seasonal effects it was not possible to be certain that differences between alternate generations were not of largely seasonal origin. Table 6.77 gives the frequency distributions of the dates of death of animals comprising suppressed and stimulated generations for DBA and C57 mice. Two arguments defend the importance of these data, however, despite the absence of information concerning possible seasonal effects; the first being that allowing for, on average, 3 weeks until conception (on several occasions successive litters from the same mother were used), 3 weeks gestation and 14 weeks average experimental age, separations of about 5 months between the peaks of distributions would be expected. Secondly, if real seasonal influences were operating further examination might show that in uniform environments such as animal houses, the alternating maternal effect might be acting as the 'tick' of a biological clock that timed seasonal reproductive cycles.

Table 6.77 Frequency distributions of death dates of 'suppressed' and 'stimulated' generations of DBA and C57 mice.

	<u>DBA</u>		<u>C57</u>	
	Suppressed	Stimulated	Suppressed	Stimulated
Month	(f)	(f)	(f)	(f)
Jan	5	3	-	-
Feb	4	-	2	10
Mar	5	1	-	-
Apr	2	-	-	5
May	-	2	4	1
June	-	2	-	-
July	-	2	3	-
Aug	-	-	1	-
Sep	-	3	2	-
Oct	-	-	2	-
Nov	3	-	3	-
Dec	3	-	-	-

The organ weights of the animals forming the pools of 'suppressed' and 'stimulated' generations, referred to in Chapter 5, were examined for differences in overall size and, by zero order, and partial, non-parametric correlations, inter-organ relationships were examined. Considering gross differences, first, it can be seen, from Table 6.78, that thymus and testis weights were significantly different between the two functional groups of animals, (as indicated by M-W 2-tail

Table 6.78 Mean thymus and testis absolute dry weights from DBA and C57 mice of alternate generations.

Maternal effect on R:-	DBA MEANS			C57 MEANS		
	Supp.		Stim.	Supp.		Stim.
		p(%)			p(%)	
Thymus	762 (21)	10.2	935 (11)	1096 (18)	NS	1047 (15)
Testis	158 (21)	2.0	145 (11)	150 (18)	0.68	162 (15)

Thymus absolute mean dry weights in mgms x 10^2

Testis absolute mean dry weights in mgms x 10

Maternal effects: Supp. = suppressive and Stim. = stimulatory.

p(%) = M-W 2-tail probability expressed as a percentage.

probabilities (%'s) between the means of groups tested) in a way that was strain dependent.

The DBA testis mean weights differed between suppressed and stimulated generations in a predicted way: lower testis weight accompanying raised adrenal function in stimulated mice.

The finding of an opposite, and much more significant, effect in strain C57 represented, potentially, a crucial difference between this, and the other, strain. The thymus weight difference in DBA's, although lacking statistical significance, was nonetheless important as it signified that, compared to C57 organs, thymuses from the former strain were more sensitive to the mediator of the maternal influence, which was argued in Chapter 5, to consist, possibly, of corticosterone, of maternal origin.

Examination of zero order, and partial, correlation coefficients provided further information on these points. Table 6.79 gives the zero order and partial non-parametric coefficients for suppressed and stimulated generations of both strains. Probabilities of difference (estimated by t tests of Z-transformed values) are given between the correlations tested. Individual correlation significances are indicated by asterisks, and superscript letters indicate (at the bottom of the table) variables controlled in the computation of partial correlations, whose significances, on account of their non-parametric nature, could not be assessed.

From the correlations of Table 6.79, it is apparent that thymus relationships with adrenal function were markedly different between suppressed and stimulated DBA, but not C57, animals, even when effects of age, testis weight and body weight had been removed (partial correlations a and b). The testis v. thymus correlations indicated that C57 thymuses were probably more sensitive to androgen than those of DBA (partial correlation c). The positive relationship (correlation d) between testis and R, after subtraction of body weight effects, was speculated as being the result of decreased corticosterone inhibition of androgen production, possibly by a mechanism common to that relaxing a restraint on corticosterone production, itself.

It was stated in Chapter 5 that corticosterone of maternal origin could be an effector of a maternal influence on the developing adrenal-pituitary axis. The organ weight results, just described, support this hypothesis; the DBA thymus sensitivity to corticosterone probably being influenced. Recent work has indicated that

Table 6.79 Correlation coefficients for DBA and C57 variables pooled from alternating generations.

	DBA			C57		
	Supp.	p(%)	Stim.	Supp.	p(%)	Stim.
R v. Thymus	-0.37 (11)	5.0	+0.36 (21)	+0.08 (15)	NS	+0.21 (18)
R v. Thymus ^a	-0.31 (9)	-	+0.34 (19)	-0.11 (13)	-	-0.04 (16)
R v. Thymus ^b	-0.26 (10)	-	+0.36 (10)	+0.10 (14)	-	-0.03 (17)
Testis v. Thymus	-0.46 (11)	NS	+0.06 (21)	-0.30 (15)	NS	-0.17 (18)
Testis v. Thymus ^c	-0.07 (9)	-	+0.13 (19)	-0.44 (13)	-	-0.19 (16)
R v. Testis	+0.33 (11)	NS	-0.17 (21)	-0.21 (15)	NS	-0.03 (18)
R v. BWT	+0.50* (13)	NS	+0.04 (21)	+0.07 (15)	2.5	-0.70** (18)
Testis v. BWT	+0.87** (11)	NS	+0.59** (21)	+0.09 (15)	NS	+0.55* (18)
R v. Testis ^d	-0.25 (10)	-	-0.24 (20)	-0.21 (14)	-	+0.59 (17)

All probabilities single-tailed: (*) 5%, (**) 1%.

Key to controlled variables in partial correlations: a = age, abs. testis wt.

b = body weight c = age, R d = body weight

cortisol acetate injections within 24 hours of birth, in rats, results in reduced stress response at 20-23 days of age, due to increased negative feedback sensitivity (Erskine et al., 1981). Genetic difference(s) have been observed, between BALB/cBy and C57BL/6By mice, with respect of hypothalamic retention of corticosterone (Eleftheriou, 1974), and a t test between reciprocal F_1 values from this work revealed that retention was lower (2-tail $p = 5\%$) in the reciprocal strain mothered by the high retention strain, C57.

It thus appeared that the reduced thymus weight, mentioned earlier, observed in mice possessing the DBA-derived allele of the Idh-1 locus, could be the result of an increased sensitivity to corticosterone similar to that noted already for AKR mice (Metcalf, 1960).

The implications of this with respect to the ald phenotype will be considered in the next chapter.

By process of elimination and also from the association of chromosome 9 effects on testis correlations, the Mod-1 and d loci were thus thought to be linked to factor(s) controlling thymus sensitivity to androgen, a character, as described earlier, that was penetrant exclusively in C57 backcrosses, unlike the Idh-1 effect which, presumably due to maternal interaction, was noted in backcross OC as well as the backcrosses to DBA. Increased sensitivity to androgen sensitivity with respect to seminal vesicle development has already been reported for C57BL/10J mice (Bartke, 1974).

A fuller discussion of the general implications of these genetic and non-genetic effects will follow in the succeeding pages.

CHAPTER 7

GENERAL CONCLUSIONS

The measurement of the major variable in this work, in vitro corticosterone productive capacity, or R, was marred by two sources of experimental error that effectively disguised each other's presence for a number of experiments; altered Coulter counting characteristics and uncontrolled bovine serum albumin purity. Although no direct evidence was obtained concerning both these faults, the indirect facts have been described, at length, in Sections 2.3 and 3.3, to the extent that knowledge and control of these sources of variation were thought to be sufficient to be certain, beyond reasonable doubt, that the use of corrected R values would not be likely to seriously mislead correct interpretation of the facts.

Consistent differences were observed, as described in Chapter 4, between DBA and the two other strains, C57 and CBA, in respect of adrenal cell corticosterone production. Furthermore, a time course experiment (Section 4.2.3) served the dual purpose of, ensuring that the phenotype examined was not biased in a way unlikely to allow all major differences between strains in adrenal corticosterone production to be expressed, and also indicating that interaction between corticosterone production with incubation length, in $F_1(N)$ cells, demonstrated traits (sub-phenotypes) that were found, singly, in cells of each parental type. This suggested that DBA and C57 adrenal cells differed in at least two ways, with respect to corticosterone productive capacity.

Dose response characteristics to ACTH appeared to be similar for all strains with the exception that $F_1(N)$ cells may possess flatter response curves, a point that will be referred to shortly.

Steroidogenesis in response to dbcAMP resulted in a similar maximal production rate as that for ACTH, with C57 cells, but was significantly greater than the latter response in $F_1(N)$ and, especially, DBA cells. From limited evidence, CBA cells were apparently similar to those of C57, in this respect. The inference from these responses to ACTH and dbcAMP, that DBA adrenals were relatively less efficient utilisers of ACTH as an initiator of steroidogenesis, compared with those of C57, will be used later in a discussion of the ald phenotype.

When the specific phenotype, R, was measured for adrenal cells derived from individual adrenal pairs (Chapter 5) it was found to display considerable functional variability among individuals of identical genotype. In some cases, as with DBA's, distributions appeared to form two virtually discrete functional groups. In this respect, they were similar to observations concerning plasma testosterone levels in mice, which oscillate between two functional levels, diurnally, to an extent dependent on genotype (Lucas and Eleftheriou, 1980). The R distributions could not be attributable to such diurnal effects as mice were killed at the same time for each experiment. As seasonal effects could not be recognised in the R observations, as sample sizes were too small, with some months, especially winter ones, being under-represented, it was not possible to discriminate between these and maternal effects (if, indeed, they were separable) as causes of the functional groupings. The fact that consistent, negative maternal influences were seen to be exerted by $F_1(O)$ and (N) females

on their young in backcrosses to both parental strains, suggested that similar effects probably exist in the absence of genetic segregation. It could be speculated that the same groupings probably serve the purposes of these different strata of control; seasonal or maternal effects tending to polarise the diurnal fluctuations towards one or other functional level.

Although genetic analysis of R distributions was made more difficult by their departure from statistical normality, the significant F values obtained for backcross:parental variance ratios, and the associations between Idh-1 and b allotypes with significantly different R distributions, indicated that genetic segregation, affecting the phenotype, was occurring. The lack of fit between a simple one gene model and observed values, even after an attempt had been made to reduce differences due to maternal influence, confirmed the hypothesis formed, from the time-course experiment, that at least two genetic differences accounted for the dissimilarity of DBA and C57 R values. Frequency distribution curves were interpreted (at the end of Section 5.8.3.4) as possibly indicating a 1:1:1:1 grouping suggestive of only a two gene difference, of a major sort, between these strains. Subsidiary influences, that revealed themselves in correlations of R with age, were observed to be linked to the Y-chromosome and the agouti locus in QD mice, and to the X-chromosome, in hybrids of DBA and C57. Analysis of the R values of F_1 's (O) and (N) suggested, however, that the latter effect contributed only slightly to their overall difference, which was thought to be, largely, the result of maternal influence. This bears out earlier observations (Treiman et al., 1970) concerning the absence of sex-linked

effects in stress responses of reciprocal hybrids of DBA/2 and C57BL/10J strains. The similarity of the CN and NC cumulative frequency distributions of R (Figure 5.8) also indicated the probable minor role of X-linkage in the modulation of R value. Small sample sizes did not permit firm conclusions being formed concerning further X- or Y-linked effects concerning target organs although they appeared to interact with Idh-1 type to produce marked differences in CN correlations between testis weight and R (Table 6.75).

Backcross QB appeared to be the only hybrid of DBA and CBA that resembled the latter strain with respect to characters such as spleen size and trend of R with age. It was possible that these were all pleiotropic effects of the Y-linked gene affecting testis size.

The presence of significantly larger spleens in ND mice, homozygous for the DBA allele of the b locus, was probably a reflection of the significantly reduced adrenal function observed in this same group of animals, in Section 5.8.1.2.2. The factor linked to this locus appeared to act largely on the adrenal, as differences in allotype specific correlations between thymus weight and R, in OD's (Table 6.71), and between thymus and spleen weights, in QD's (Table 6.76), were not found to be significantly different by t test. It was conjectured that this factor may have been responsible for the difference in a time-independent character between DBA and C57 adrenal cells, that was thought to consist of general steroid synthetic capacity (Section 4.2.3).

Although the significantly lower R values of adrenal cells

from animals possessing the C57-derived Idh-1 allele were thought to be associated with redder adrenal colour (lower lipid content) and thus be the expression of an ald locus, this conjecture will be required to be proved by using a less arbitrary measure of lipid content such as one of those used already to examine the ald phenotype (Doering *et al.*, 1973; Stylianopolou and Clayton, 1976a).

Equally, no definite conclusions could be reached concerning the exact nature of the Idh-1- and Mod-1-linked effects on thymus size except that they exerted opposing influences; the DBA allele being associated with smaller thymus size in DBA backcrosses, for the former locus, and with larger organs in C57 backcrosses, for the latter genetic marker. Although distinction of function was unclear, between these two marker-linked effects, particularly with respect to correlations between R and thymus weight (Table 6.71), the evidence presented in Chapter 6, on balance, was thought to indicate that the Idh-1-linked factor controlled sensitivity to corticosterone, whereas Mod-1 type appeared to influence relationships between testis weight (and presumably androgen) and other organs. This conclusion was not predictable in terms of facts already known with respect to C57 relative androgen insensitivity, in such characters as kidney sexual dimorphism (Bartke and Shire, 1972), sex drive (McGill and Manning, 1976) and renin activity induction in the submandibular gland (Wilson *et al.*, 1978). The mapping of the latter effect to the Rnr locus on chromosome 1 might even suggest an androgen-related function for the Idh-1-linked effect.

On the other hand, the importance of androgen in modulating thymic function has been noted already, particularly with respect to

autoimmune disease development (Steinberg et al., 1980). If the relative adaptive importance of this function approached that proposed for the increased sensitivity of seminal vesicles of C57, compared with DBA, mice (Bartke, 1974), such hypersensitivity to testosterone might be expected to express itself with respect to the thymus, as well, in C57's. Nearly all previous observations concerning androgen sensitivity have been made on sublines C57BL/6 and C57BL/10 which have been found to possess smaller testes than C57BL/Tb, the subline of the present work, and C57BL/Ka (Shire and Bartke, 1972; Shire, 1981) due to an early split in the C57BL lineage. This could mean that this strain has evolved a certain degree of polymorphism with respect to androgen sensitivity among its sublines. These arguments, combined with the observation of corticosterone hypersensitivity in another strain displaying the ald phenotype (Metcalf, 1960), as well as the inhibition of radiation-induced leukaemia in C57BL mice by testosterone (Wasi and Block, 1961), suggested the roles inferred for the Idh-1- and Mod-1-linked effects, in Chapter 6, were at least possible. Although the proposed hypersensitivity of DBA lymphoreticular tissue to corticosterone may have some bearing on the reduced incidence of autoimmune phenomena in NZB/DBA hybrids, it is probable that the apparently dominant inheritance of gene(s) controlling DBA testis size could result in such hybrids possessing higher levels of protective androgen. This latter explanation was strengthened by the very marked differences (of sex-linked or maternal origin) observed in correlations between testis weight and spleen or thymus weight, for F_1 's (O) and (N) in Section 6.8.1. Such reciprocal F_1 differences may have accounted for the difference in severity of autoimmune

disease observed in reciprocal crosses between DBA/2 and NZB mice (Raveche *et al.*, 1978). Future examinations of the lymphoreticular systems of DBA/2 and C57BL mice should measure the extents of binding of radio-labelled corticosterone and testosterone to lymphatic structures, as well as observe strain differences in involution responses to injections of a particular steroid. This would provide concrete evidence concerning the very different means by which the two strains undergo relatively similar rates of thymic involution.

Although differences have been observed between C57BL and AC (adrenal lipid depleted strain) with respect to dehydrogenase activities in the phosphogluconate oxidation pathway (Molne *et al.*, 1969), and adrenal mitochondrial isocitrate dehydrogenase and malic enzyme activities have been shown to increase upon ACTH stimulation in the rat (Laury and McCarthy, 1970), adrenal transplantation (Taylor *et al.*, 1974) and chimaera (Shire, 1979a) studies using the ald-1 gene have suggested it exerts its primary effect at a site outwith the adrenal.

A highly speculative hypothesis concerning the cause of the ald phenotype can be based on the assumption that the Idh-1 effect on thymus size was the result of altered corticosterone sensitivity, and also that the effect, linked to the same marker, on R (and adrenal lipid content) emanated from an ald locus. It could be argued that mutations producing ald phenotypes would be more likely to occur, and result in polymorphisms that did not seriously impair fitness, if the locus responsible for their cause also ensured that target organ sensitivity was also appropriately modified. The primary site of action of ald mutations could be the hypothalamus, where increased

sensitivity to corticosterone (by, conceivably, the variety of means observed for dexamethasone-resistant lymphoma lines by Sibley and Tomkins, 1974) would suppress ACTH release. It has been shown in the case of AKR's that their lipid depletion is remediable by injection of high doses of ACTH (Arnesen, 1956). ACTH insufficiency would be a possible explanation of the difference observed between the dbcAMP and ACTH responses of DBA cells, referred to earlier in this chapter.

Provided corticosterone target tissue such as the thymus was also endowed with such increased sensitivity to corticosterone, the reduced corticosterone output by the hypofunctional adrenals would have little effect on fitness.

In contrast, the presumed high adrenal corticosterone production by strain BALB/cBy mice (Eleftheriou and Bailey, 1972; Shire, 1979a) may be due to hypersecretion of ACTH due to the relative insensitivity of these animals' brains to feed-back inhibition by corticosteroids (Hawkins et al., 1975; Sakellaris et al., 1976) probably due to reduced hormone binding, especially in the hypothalamus (Eleftheriou, 1974). This may be counterbalanced, in the same way that DBA hypersensitivity has been speculated to be, by brain insensitivity extending to target tissue such as the spleen, in BALB/c's, which appears to be less sensitive to growth restricting influences, in this strain, than in C57BL's (Mos, 1976).

The induction of the ald phenotype by the androgen surge at puberty could be explained, within such a model, if glucocorticoid receptors, in strains such as DBA, were capable of also binding androgen, in a similar way as that observed in rat skeletal muscle (Mayer and Rosen, 1975). The latter class of steroid, being the dominant one in strain DBA, could be envisaged as, potentially, exerting a strong negative influence on the pituitary-adrenal axis by cross-binding with corticosterone receptors in the hypothalamus. It is probable,

however, that lipid depletion would be induced in the adrenal by more than just a reduced ACTH output as blockade of this hormone with specific antibodies, for a week, in rats, resulted only in reduced corticosterone productive capacity by isolated adrenal cells; hypophysectomy reducing adrenal lipid content (-86%), as well (Rao et al., 1978). The re-accumulation of adrenal lipid in AC mice after injection with cortisone acetate (Arnesen, 1964) could have resulted from negative feed-back on late steps in corticosterone synthesis in the adrenal (Mulrow, 1972), rather than inhibition of pituitary ACTH hypersecretion.

If cross-binding between steroid classes and their respective receptors transpired to be a general phenomenon, due to fitness advantages mentioned in the last chapter, the predominant steroid of strain C57, corticosterone, may assert its dominance by binding with testosterone receptors in the brain in an analogous fashion to its androgenic effect in the submandibular glands of normal mice, but not in testicular feminised animals (Sato et al., 1981). C57BL testis function may, conceivably, be inhibited by corticosterone in an analogous way to the proposed mechanism by which testosterone suppresses adrenal function in DBA's.

Such speculation may not prove to be the correct explanation of adrenal lipid depletion but, even assuming the operation of an androgen-sensitive locus that was distinct from the factor that appeared to be associated with sensitivity of the thymus to steroids, particularly corticosterone, the finding of Chapter 5, that the DBA ald locus may map to the same chromosome as that of AKR-related strains, may still be best explained by an extra-, rather than an intra-, adrenal primary cause. The segregation in adrenal lipid

contents observed in the backcross of F_1 (AC x DBA) to DBA (Doering et al., 1973) could have arisen as a result of segregation of what must be a modifying influence; androgen production. Pre-pubertal castration of such backcross animals, followed by androgen injection (possibly taking account of individual differences in metabolism) might, conceivably, result in an absence of such phenotypic segregation. It is probable that more information, concerning the main cause (if there is such a one) of the ald phenotype, has to be obtained before it proves possible to decide whether or not the phenotypes observed in AKR lines and DBA mice are caused by a common genetic defect. Until this has been achieved it may be misleading to regard the phenotype as a 'defect' confined to single ald loci instead of perceiving it as a 'normal' variant whose expression is contingent on the actions of other co-adapted genes. Selection for proneness to certain pathologies during the histories of certain strains could be envisaged as resulting in adrenal function variations, such as the hypofunction of AKR's. It is likely that 'normalising' selection would be exerted at the same time, however, to ensure that the adrenal variant (amongst others, probably) did not result in drastically reduced reproductive fitness.

Maternal influence was observed in the present work to result in strains O, ND and NC having higher adrenal lipid contents (yellowish adrenal colour) than those found in respective reciprocal strains, N, OD and OC. A t test applied to cholesterol ester levels obtained in a previous work (Doering et al., 1973) indicated that the equivalent of O mice, in the present work, had significantly higher cholesterol ester scores (2-tail $p = 0.01$) than those of the equivalent of

strain N. Another study, measuring lipid/cortex ratios (Wahlsten and Anisman, 1975), obtained ratios for the F_1 corresponding to O that were three times those of the strain analogous to N. It was proposed in Chapter 5 that the maternal influence, observed on corticosterone productive capacity (R), was due, in part, to altered cholesterol ester levels. The ACTH dose response curve was found to be flatter for $F_1(N)$ than for either parental strain, as mentioned earlier. This was thought to be due to reduced sensitivity to ACTH which was thus concluded to be released in smaller amounts in this F_1 compared to its reciprocal, strain O. It was thought, therefore, that the maternal influence on R was the result of enhanced feed-back sensitivity of the brain to corticosterone, due to exposure to higher levels of maternally originated corticosterone, in early life, compared to its reciprocal counterpart, $F_1(O)$. The pervasiveness of maternal effect(s), among the organ and body weights, observed in Chapter 6, as well as their interaction with foetal genotype, suggested that they may be more than mere ontogenetic 'noise'; perhaps possessing positive adaptive advantages. It was possible that such effects were exceptional; being the result of crossing strains that were at extreme ends of the spectra of testis and adrenal activities.

The generality of the phenomenon, however, has been indicated by observations of marked maternal influences altering expressions of; audiogenic seizure proneness (Beck and Gavin, 1976), autoimmune disease (Simpson, 1973; Raveche et al., 1978), open-eye mutants (Nguyen-Trong-Tuan et al., 1971) and response to amphetamines (Jori and Rutczynski, 1978) in a number of different strains of mice. With respect to human disease, cord blood cortisol concentration

has been proposed to be of predictive value with respect to illnesses, particularly of autoimmune type (Levina, 1980). The variable penetrances of various types of adrenal-related human diseases may be caused by negative maternal effects; the incidence of essential hypertension in offspring of affected people having been observed to be much lower than might be expected (Kaplan et al., 1978). A kindred of individuals (a boy, his mother and his grandmother) with dexamethasone-suppressible hyperaldosteronism displayed degrees of severity that alternated between successive generations (Ganguly et al., 1981). Variable penetrance of Van der Woude type cleft palate has also been observed, in humans (Shprintzen et al., 1980), and a pedigree compiled by these authors gave the following proportions of individuals with lip pits in generations linked by maternal descent: 3/6 (50%), 9/14 (64%), 7/7 (100%) and 4/6 (66%). Although the incidence of affected individuals, in the first generation, did not alternate in magnitude, as it did in subsequent generations, this could have resulted from a spontaneous mutation occurring half-way through the reproductive lives of their parents, about whom nothing was known. Permanent maternal influences upon ontogeny may also explain the differing predispositions to neoplasia of people born in winter and summer months, cited in the formulation of a theory of cancer aetiology dependent on abnormal hypothalamus function (Tromp, 1974).

The apparent ubiquity of maternal influences might suggest that, far from being harmful, they may, in fact, have become 'harnessed', genetically, to adaptive advantage, by a mechanism that is readily comprehensible using a model of development that places much emphasis on environmental interactions. In such a model, complex gene interactions, occurring during the development of a particular character, have been conceptualised as exerting a canalising effect that, to a

greater or lesser extent, prevents environmental influences from subverting development out of one pathway into another (Waddington, 1957). If susceptibility to an environmentally-induced anomaly (phenocopy) is selected for, the phenocopy becomes easier and easier to produce, until, in certain cases, it occurs spontaneously in the absence of any environmental trigger. Among the many facets of the pre- and post-natal environment, none might be expected to act as triggers with more power than maternally-derived hormones. It is thus conceivable that 'phenocopies', consisting, perhaps, of individuals with altered functional states of endocrine and other tissues, might be produced that were as permanent as if they had been entirely genetically produced. If such 'phenocopies' offered some adaptive advantage or increased reproductive success, selection would then be expected to exist that favoured those individuals whose genes (by their canalising influence) permitted such phenocopies to arise.

Using an example from the present work, high concentrations of maternally-derived corticosterone, at the beginning of mouse evolution, may have interacted with fetuses (or weanlings) to produce a few phenocopies that consisted of adults with reduced adrenal corticosterone productive capacity. In the course of natural selection, genes that increased the occurrence of these phenocopies would tend to increase in frequency, on account of the increased fitness enjoyed by such animals, perhaps as a result of being better prepared to face crowded breeding conditions or changing season. Eventually the 'phenocopy' response to maternal hormone level would become constitutive, but not to the extent that the 'phenocopy' was produced, spontaneously, in the absence of the hormone trigger. A new genetically

determined adaptive response will have emerged; the ability to switch pituitary-adrenal development into a lower functional state whenever high levels of maternally-derived corticosterone were encountered. In theory a bimodal adult functional distribution could be ensured by the development of one 'phenocopy' only; one functional state representing a 'normal' one. It would be unlikely, however, that a hyperfunctional phenocopy, produced in response to low maternal corticosterone concentrations, would not tend to become constitutive, by the same means, and for the same reasons, as that producing hypofunction.

Although such maternal effects have been observed before with respect to plasma stress responses (Treiman et al., 1970), where they were conjectured to exert a buffering or moderating influence that appeared to oppose the expression of extreme genotypes, this interpretation was only partially true for the present results concerning adrenal corticosterone production. Although maternal effects have been observed to operate negatively with respect to R, it has been proposed that they depend on the phenotype (functional state) of the dam, and not the genotype, as evidenced by the differences between reciprocal backcrosses. The conclusion reached was that, in fact, phenotypic variability was increased (considering all reciprocal backcrosses), and not reduced, by maternal influences, and such increased diversity, occurring in the absence of increased genetic variability, may be of evolutionary advantage, especially with respect to genetic load considerations. In circumstances of reduced genetic segregation, such as inbred populations, or their F_1 hybrids, such phenotypic diversity could possess significant survival

value. In the presence of genetic segregation, a further type of 'phenocopy' might prove advantageous, especially with respect to seasonal environmental changes. This 'phenocopy' would be lethal so that intra-uterine resorption occurred in response to a certain level of maternally-derived hormone. This level would depend on the current functional state of the dam, which could reflect seasonal alterations, overlayed on her genetically- and maternally-defined constitution. If the occurrence of such lethal phenocopies became constitutive, in the course of evolution, it would be possible to obtain genotypes that possessed survivals that varied according to the time of year in which they were born. Preliminary data, collected over two years, in the same animal house as the mice used for the rest of this work were bred, indicated that significant seasonal fluctuations existed for the frequencies of alleles of both the b and the d loci, especially in OD, compared to ND, mice. Embryonic mortality, associated with the shadow coat colour gene in minks, has been suggested to be partially controlled by the day length experienced by the dam, during pregnancy (Belyaev and Zhelezova, 1978). It is possible that preferential survival of different genotypes at different times of the year could be advantageous in optimising the maternal resources, with respect to those animals most genetically suited to being born into the prevailing environment.

A greater appreciation of the potential existence of maternal effects may enable the multi-factorial theories, used, currently, to explain many human regulatory disorders with variable penetrance, to be revised in favour of simpler hypotheses. The potential may also exist for manipulation of the maternal and neonatal environments to minimise undesired genetic expression.

Although more observations will be required to test many of the conclusions reached in this work, it is clear that considering gene effects, in isolation, without referring to all conceivable interactions with the environment, may be particularly misleading in any assessment of the pathological, and evolutionary, significance of an endocrine variant.

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